THE INFLUENCE OF PREVIOUS EXPOSURE AND SOIL TYPE ON THE DEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN SOIL

BY

JACKI BL HEAMAN

A Thesis
Submitted to the faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Soil Science
University of Manitoba
Winnipeg, Manitoba

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FACULTY OF GRADUATE STUDIES

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ABSTRACT

Heaman, Jacki B.L. M.Sc., The University of Manitoba, February, 1999. The Influence of Previous Exposure and Soil Type on the Degradation of Polycyclic Aromatic Hydrocarbons in Soil. Major Professor; Dr. David L. Burton.

Soil microbial communities have the potential to degrade PAH contaminants (Kanaly *et al.*, 1997; Pothuluri and Cerniglia, 1994; Sims and Overcash, 1983) but the microenvironment may be unsuitable for activation of the required degradation pathways (Grosser *et al.*, 1991; Herbes and Schwall, 1978; Morgan *et al.*, 1993). Three PAH compounds were chosen for a study which examined the influence of slope and soil type, previous hydrocarbon contamination, and environments where ligninolytic activity would be selected for, on the degradation of PAHs in a soil environment.

The influence of landscape position and soil type on the ability of the indigenous microbial community to degrade a persistent contaminant such as B(a)P was examined. A degradation study was performed that utilized soil samples representative of four slope positions located in four soil associations found in a transect across Manitoba. ^{14}C-labeled B(a)P in a diesel fuel stock solution was added to each of the 16 soils. Limited degradation was observed (< 2 %).

In a second study soils from two previously contaminated sites were used for a laboratory based degradation study. ^{14}C-labeled anthracene and B(a)P were added to the soil in a diesel fuel stock solution. Anthracene was degraded in all seven soils

examined. B(a)P degradation experienced a lag time of several weeks before three soils were able to produce significant amounts of $^{14}CO_2$ from the B(a)P. At each of the two sites the soils with the highest level of hydrocarbon contamination had an increased ability to degrade the PAHs and the simpler compound was degraded before the more complex.

The final objective of this study was to determine if a previously uncontaminated forest soil, where ligninolytic activity is anticipated to be high, would result in enhanced PAH degradation. The LFH, Ahe, and Ae horizons of a Luvisolic soil were chosen for degradation study using ^{14}C-labeled naphthalene, anthracene, and B(a)P. Degradation levels of naphthalene (2.9 to 3.3 %), anthracene (2.7 to 3.1 %), and benzo(a)pyrene (0.4 to 0.5 %) were extremely low. This study found that soils with previous exposure to hydrocarbon contamination have an increased ability to degrade PAH compounds, including B(a)P.

FORWARD

The following thesis was prepared using the manuscript format outlined in the Guide to Thesis Preparation for Graduate Students in the Department of Soil Science. All of the manuscripts presented in the thesis (Chapters 3, 4, and 5) will be submitted for publication to refereed journals. The manuscripts will also include a co-author, Dr. David L. Burton, who is also the major professor and advisor.

CHAPTER 1

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants. Their carcinogenic and persistent nature has lead to various PAHs being listed as priority pollutants. Incomplete combustion of organic-carbon based materials during industrial processing, such as petroleum refining, coal gasification, and coal liquification, are responsible for the generation of PAHs (Miller *et al.*, 1988). Improper disposal of petroleum based products at production plants and at facilities using these substances has created soil and water pollution problems (Mueller *et al.*, 1989). In the environment PAHs create potential health problems and remain persistent throughout natural ecosystems (Pothuluri and Cerniglia, 1994). The structural stability of these compounds and the tendency to sorb to soils and sediment have resulted in great difficulties remediating PAH contaminated sites. Unsubstituted PAHs are extremely resistant to catabolism by microorganisms, and are only known to degrade in the presence of an adequate oxygen supply (Manilal and Alexander, 1991; Pothuluri and Cerniglia, 1994). The ultimate goal of the degradation of organic contaminants as a remediation procedure is to produce less harmful intermediate compounds and the eventual production of carbon dioxide and water (Wilson and Jones, 1993).

Soil microbial communities have the potential to degrade PAH contaminants (Kanaly *et al.*, 1997; Pothuluri and Cerniglia, 1994; Sims and Overcash, 1983) but the

microenvironment may be unsuitable for activation of the required degradation pathways (Grosser *et al.*, 1991; Herbes and Schwall, 1978; Morgan *et al.*, 1993). If the factors influencing PAH degradation can be determined, then altering the soil conditions may induce the expression of the enzyme pathways responsible for the breakdown of PAHs. Naphthalene, anthracene, and benzo(a)pyrene [B(a)P] were three PAH compounds chosen to examine a number of factors that may be influencing the degradation of PAHs in a soil environment.

The main hypothesis is that soil microbial communities have the potential to degrade PAH contaminants but the conditions of the microenvironment may be unsuitable to activate the required degradation pathways. The three experiments included in this study examined the influence of landscape position and soil type, previous hydrocarbon contamination, and ligninolytic systems on the degradation of PAHs.

The objective of the first study was to examine the influence of landscape position and soil type on the ability of the indigenous microbial community to degrade a persistent contaminant, B(a)P. Soil samples taken along four catena sites at four different slope positions provided populations of microorganisms from a range of microenvironments. Slope position and climate influence local hydrology and soil properties as well as the composition of the indigenous microbial community at a particular location. Differences in the organic matter content and the water holding capacity of soil will determine the availability of B(a)P to the microorganisms and the composition of the microbial community.

The second experiment was designed to determine if hydrocarbon contaminated sites had an adapted microbial community with an increased ability to degrade anthracene and B(a)P. Soils that have been contaminated for a period of time may have a microbial community that has adapted to the presence of the pollutants or that can utilize the hydrocarbon contamination as an additional source of carbon. Soils from two different contaminated sites were used to determine the kinetics of PAH degradation. The rate of degradation of these two PAHs was examined to determine if size, activity, or diversity of the biomass could act as biological indicators of a soil's potential to degrade PAHs.

The third experiment was to determine if a Luvisolic soil, where ligninolytic activity is anticipated to be high, would result in enhanced degradation of PAH compounds. Lignin degradation requires nonspecific enzyme systems that have been found to degrade xenobiotics, such as B(a)P, as a beneficial side reaction (Hammel, 1992). The top three horizons of a Luvisolic soil (LFH, Ahe, and Ae) from the Riding Mountain area were collected for use in a lab based degradation study. ^{14}C-labeled naphthalene, anthracene, and B(a)P were added to the soil horizons in a diesel stock solution. Three PAH compounds were chosen for this experiment to determine if the complexity of the PAH compound itself influenced the ability of the microbial community to degrade PAHs.

CHAPTER 2

Literature Review

2.1 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs have become widespread pollutants that remain persistent in the environment. In Canada many of these compounds have been classified as toxic and are placed on the Priority Substances List by the Minister of the Environment and the Minister of Health (Government of Canada, 1994). There are 16 PAHs that have been labeled priority pollutants in the USA (Wilson and Jones, 1993).

PAHs form a family of benzene derivatives consisting of adjoined six membered rings of carbon atoms that vary in complexity from simple two ringed structures like naphthalene to condensed multi-ringed substances such as benzo(a)pyrene, Figure 2.1 (Pothuluri and Cerniglia, 1994). Low molecular weight (LMW) PAHs have less than four rings, while four rings and greater are considered to be high molecular weight (HMW) compounds (Government of Canada, 1994). These ringed compounds can be found in linear, cluster, or angular arrangements, with increasing stability from linear to angular (Sims and Overcash, 1983). The stability of these aromatics is greatly increased as the number of rings in the structural makeup of the compound is increased (Bumpus, 1989). PAHs in general are insoluble in aqueous environments and become more insoluble as the number of rings increase (Wilson and Jones, 1993). The octanol-water

partition coefficient (K_{ow}) and the melting point of PAHs increase with ring number while the vapour pressure and the Henry's law constant decrease (Government of Canada, 1994; Schwarzenback *et al.*, 1993).

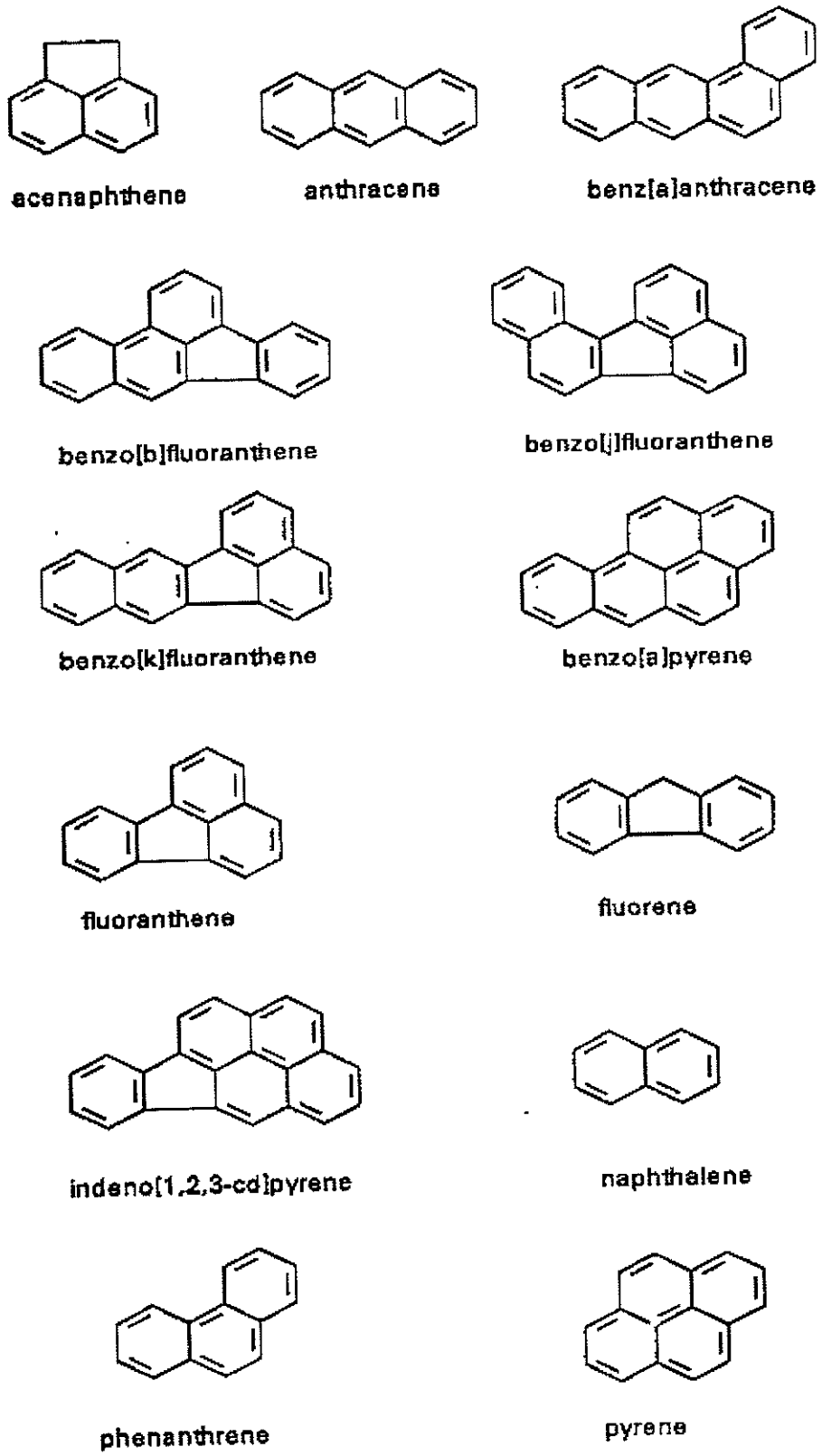


Figure 2.1 Chemical structures of PAHs (Government of Canada, 1994).

2.1.1 Source

PAHs are the result of burning organic based materials where the composition of the aromatics produced are temperature dependent (Wilson and Jones, 1993). Forest fires are the largest natural source of PAHs, while anthropogenic sources of PAHs result from the processing of organic based products and include industrial activities such as, petroleum refining, coal gassification, asphalt production, and aluminum smelting (Government of Canada, 1994). Anthracene oil and creosote are commonly applied wood pesticide treatments which have led to PAH contamination within the environment. Improper disposal of petroleum based products at production plants and at facilities using these substances has created soil and water pollution problems (Mueller *et al.*, 1989). Incomplete burning of fossil fuels has also contributed to the increase of PAH levels in the environment over the last century (Pothuluri and Cerniglia, 1994). Table 2.1 lists the concentration of PAH compounds that were detected at a number of contaminated sites. The level of contamination at the wood-preserving site indicates that concentration of PAHs is higher in the sub-soil than in the surface-soil. The range of PAH contamination detected at the creosote and gas works sites display the variability of the hydrocarbon contamination.

Table 2.1 Concentrations of PAHs at contaminated sites (Wilson et al., 1993).

PAH	Wood-Preserving		Creosote Production		Wood Treatment	Coking Plant 1	Coking Plant 2	Gas Works	
	Surface-soil	Sub-soil	Mean	Range				Mean	Range
Naphthalene	1	3925	1313	<1-5769	91.8	56	59		
1-Methyl naphthalene	1	1452	901	<1-1617			87		
2- Methyl naphthalene	1	623	482	6-2926			112		
2,6-Dimethyl naphthalene	2	296							
2,3-Dimethyl naphthalene	1	168							
Acenaphthalene	5	49	33	6-77			187		
Acenaphthene	7	1368					29	2	0-11
Fluorene	3	1792	650	49-1294	620	7	245	225	113-233
Phenanthrene	11	4434	1595	76-3402	1440	27	277	379	150-716
Anthracene	10	3037	334	15-693	766	6	130	156	57-295
2-Methyl anthracene	14	516							
Fluoranthene	35	1629	682	21-1464	1350	34		2174	614-3664
Pyrene	49	1303	642	19-1303	983	28	285	491	170-833
2,3-Benzo(b)fluorene	8	288							
Chrysene	38	481	614	8-1586	321	11	135	345	183-597
Benzo(a)pyrene	28	82			93.7	14		92	45-159
Benz(a)anthracene	12	171			356	16	200	317	155-397
Benzo(b)fluoranthene & Benzo(k)fluoranthene	38	140						260	108-552
Dibenz(ah)anthracene					10.1	2		238	152-446
Indeno(123cd)pyrene	10	23						2451	950-3836
								207	121-316

2.1.2 PAH Content in Petroleum Products

Petroleum products are produced by a fractional distillation procedure that results in complex mixtures of hydrocarbon compounds. The source of the petroleum and the temperature of the fractionation procedure affect the composition of each final product (Gillespie *et al.*, 1986). Creosote contains 17 PAHs (Government of Canada, 1994) which account for approximately 85 % of creosote by weight (Mueller *et al.*, 1989; Wilson and Jones, 1993). The remainder of creosote is made up of a phenolic fraction (10 %) and a heterocyclic fraction (5 %). Crude oil has a low PAH content (1.8 %), most of which is 2 and 3 ringed PAHs, and the major component of crude oil is saturates (74 %) (Kanaly *et al.*, 1997). Diesel fuel contains hundreds of alkane, monoaromatic, and PAH compounds. Alkanes make up the majority of diesel though PAH content can represent a large fraction of diesel (10-30 %) (Block *et al.*, 1991).

2.1.3 Health Concerns

PAHs may enter the body by inhalation, ingestion, or through contact with skin. Organisms try to detoxify and excrete organic compounds that are foreign, but while trying to detoxify PAHs, such as B(a)P, they can be metabolically activated. B(a)P is oxidized in the bay region to 7,8-diol-9,10-epoxide which becomes covalently bound to DNA (Phillips, 1983). Epoxide intermediates formed in the bay region are considered to be the carcinogenic form of PAHs that pose a risk to human health (Government of Canada, 1994) and may result in the formation of tumors (Sims and Overcash, 1983).

Acute and chronic toxicity studies have been performed in order to assess the potential threat that PAHs may pose to humans. From the information in Table 2.2 a

direct relationship between ring number and toxicity of an individual PAH can be established.

Table 2.2 Acute and chronic toxicities of PAHs (Sims and Overcash, 1983).

PAH	Ring Number	Acute Toxicity LD50 (oral rat or mouse) (mg/kg)	Chronic Toxicity Carcinogenicity in animals (mg/kg)	Mutagenicity (Ames Assay)
Naphthalene	2	1 780	negative	negative
Anthracene	3	unknown	3 300	negative
Phenanthrene	3	700	negative	negative
Benzo(a)anthracne	4	unknown	2	positive
Benzo(a)pyrene	5	50	0.002	positive

2.1.4 Guidelines and Restrictions

The Canadian Council of Ministers of the Environment (CCME) had established the Interim Canadian Environmental Quality Criteria for Contaminated Sites that outlined acceptable levels of individual PAHs that could be present in soil and water (CCME, 1991). In 1997 the acceptable values for naphthalene and benzo(a)pyrene were reduced based on scientific documentation. These new values outlined in the Recommended Canadian Soil Quality Guidelines, March 1997 (CCME, 1997) can be seen in Table 2.3.

Table 2.3 Chemical properties and CCME guidelines for naphthalene, anthracene, and benzo(a)pyrene.

Property	Units	Naphthalene	Anthracene	Benzo(a)pyrene	Reference
Ring Number		2	3	5	(Government of Canada, 1994)
Molecular Formula		$C_{10}H_8$	$C_{14}H_{10}$	$C_{20}H_{12}$	(Schwarzenback et al., 1993)
Molecular Weight		128.12	178.2	252.3	(Schwarzenback et al., 1993)
Melting Point	°C	80.6	217.5	159.8	(Schwarzenback et al., 1993)
Water Solubility	mg/L	31.7	0.045	0.0038	(Government of Canada, 1994)
log K_{ow}		3.36	4.54	5.91	(Schwarzenback et al., 1993)
Henry's Law Constant		0.018	0.294/0.00091	5×10^{-5}	(Schwarzenback et al., 1993)
Vapour Pressure at 25°C	mPa	11960	25	0.37×10^{-6}	(Government of Canada, 1994)
Ionization Potential	eV	8.1	7.4	7.2	(Hammel, 1992)
Half Life Surface Water		High 20 days Low 12 hours	High 1.7 hours Low 0.58 days	High 1.1 hours Low 0.37 hours	(Howard et al., 1991)
Half Life Ground Water		High 258 days Low 24 hours	High 2.52 years Low 100 days	High 2.9 years Low 114 days	(Howard et al., 1991)
Half Life Soil		High 48 days Low 16.6 days	High 1.26 years Low 50 days	High 1.45 years Low 57 days	(Howard et al., 1991)
CCME Guidelines, 1991*	mg/kg	0.1/5/50/50	nd	0.1/1/10/10	(CCME,1991)
CCME Guidelines, 1997*	mg/kg	0.1/0.6/22/22	nd	0.1/0.7/0.7/0.7	(CCME, 1997)

* 1991 interim remediation criteria for soil was based on 3 types of land use: agricultural / residential-parkland/ commercial/industrial
1997 soil quality guidelines separated land use into four divisions: agricultural / residential-parkland / commercial / industrial

2.1.5 Naphthalene

Naphthalene is the simplest of the PAH compounds and is a white solid at room temperature. It consists of two rings and has the lightest molecular weight of the PAHs. The octanol-water partition coefficient is relatively low and the water solubility is relatively high. The main removal mechanisms for naphthalene from contaminated soil is thought to be volatilization and biodegradation, though both these processes have an inverse relationship with soil depth. Naphthalene is thought to have a limited ability to sorb to soil and to rapidly desorbed. The mobility of this compound through a soil profile can lead to ground water contamination. Biological transformation of naphthalene can lead to complete metabolic breakdown or to the formation of intermediate degradation products (Figure 2.2 and 2.3) (CCME, 1997).

The common factor between the bacterial (Figure 2.2) and the fungal (Figure 2.3) pathways is that they both require oxygen for the initial transformation of the ring structure. Fungi add a single atom of oxygen across the carbon-carbon double bond while bacteria add two oxygen atoms during the initial oxidation step. The conditions of the soil environment will influence the bacterial and fungal composition of the microbial community and therefore will determine which of the pathways will dominate PAH degradation. The aerobic requirement of the soil microbial community to degrade PAHs is further discussed in section 2.3.1 and the enzymes involved in the first oxidation step are outline in section 2.2.3.1.

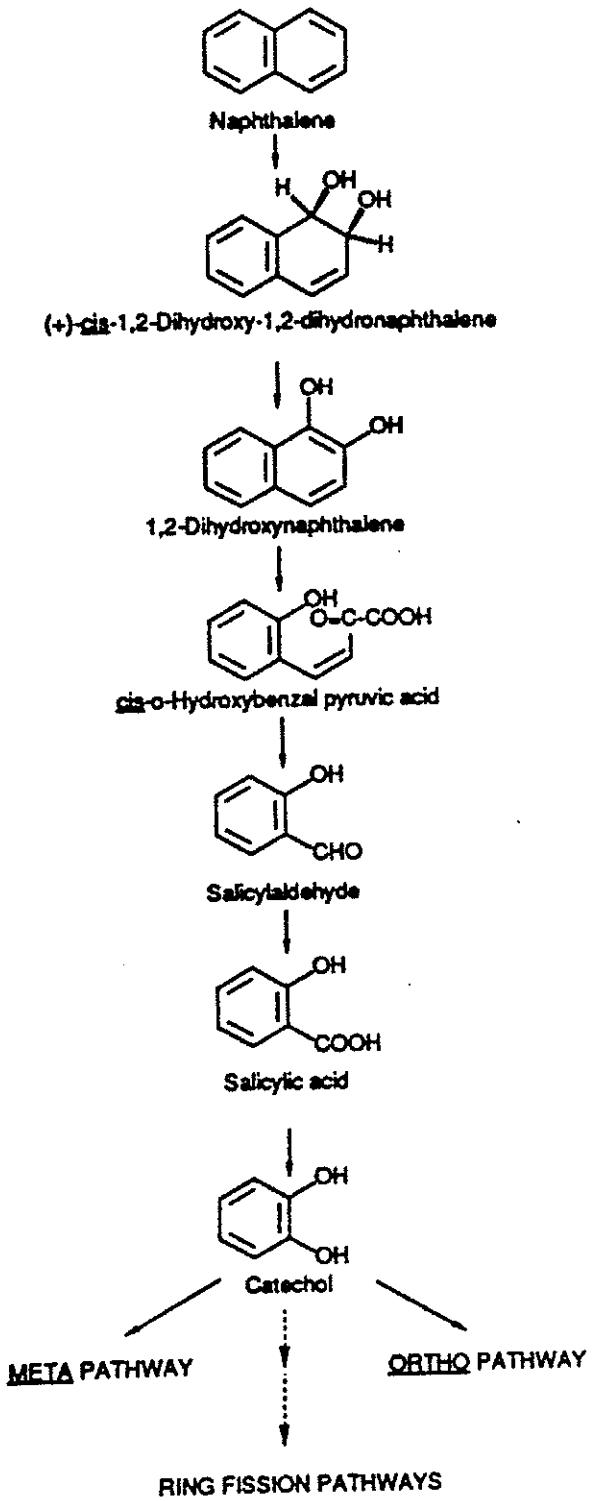


Figure 2.2 Bacterial degradation of naphthalene (Pothuluri and Cerniglia, 1994).

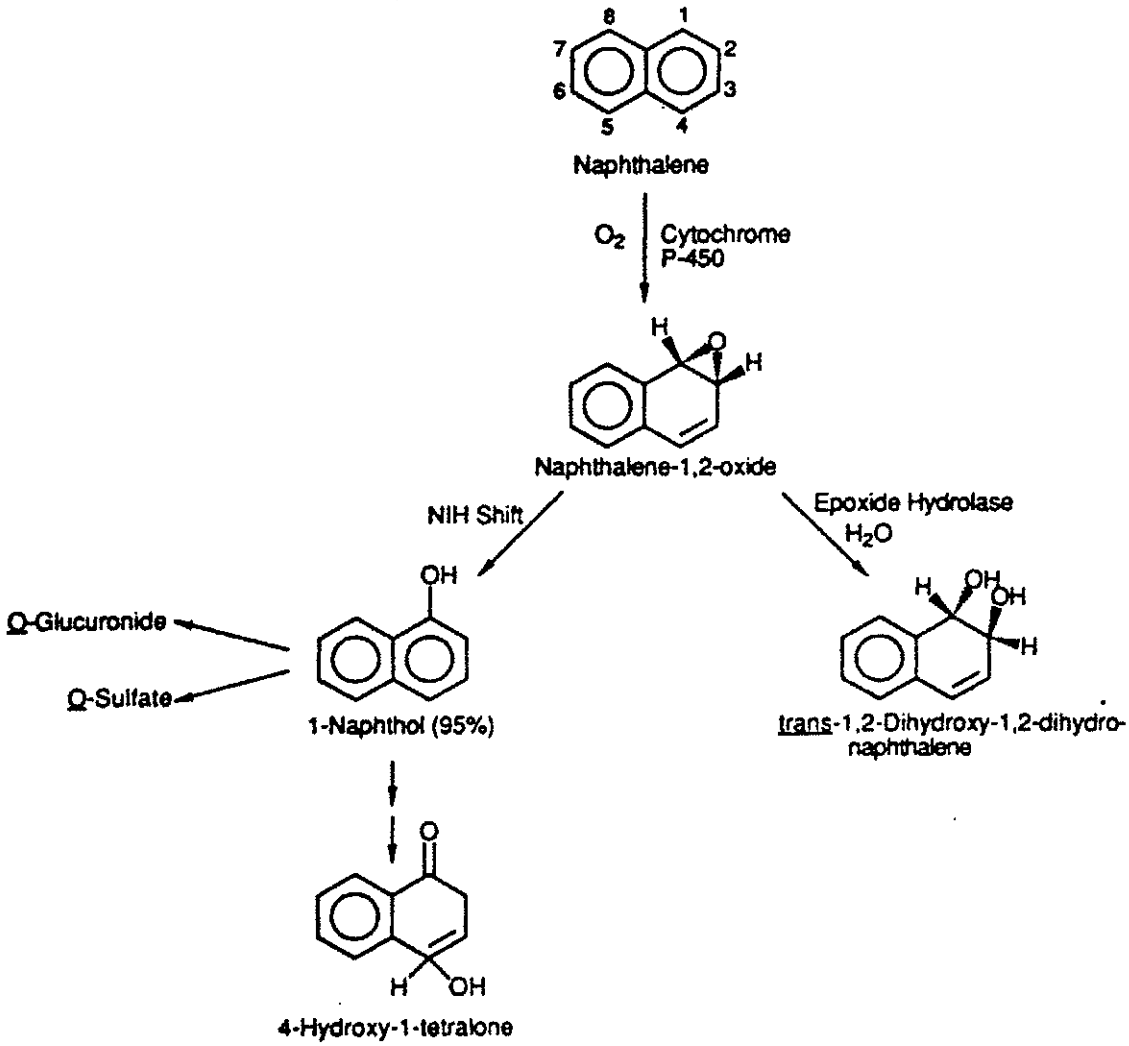


Figure 2.3 Fungal transformation of naphthalene (Pothuluri and Cerniglia, 1994).

2.1.6 Anthracene

Anthracene is a three ringed PAH of intermediate complexity and stability. The bacterial and fungal degradation pathways for anthracene are outlined in Figure 2.4 (Pothuluri and Cerniglia, 1994). Similar to naphthalene degradation, the fungal and bacterial metabolic pathways for anthracene differ by the number of oxygen initially added across the carbon-carbon double bond. It is important to understand the breakdown of simpler PAH compounds such as anthracene since their structures are contained in the more complex carcinogenic PAHs such as B(a)P. Degradation of the more complex compounds results in compounds resembling the simpler PAHs. Anthracene and its degradation intermediates are not carcinogenic (Pothuluri and Cerniglia, 1994). Once the initial ring fission has taken place, the remainder of the degradation intermediates resemble those found in the naphthalene degradation pathways previously shown in Figure 2.2 and 2.3.

Monomethyl derivatives of anthracene have not been found to be mutagenic and carcinogenic; although if methyl substitution has taken place at both the 9- and 10-positions, it has been found to cause an increase in the potential for anthracene to be a mutagenic tumor initiator (Muncnerova and Augustin, 1994).

2.1.7 Benzo(a)pyrene

The low vapour pressure and high octanol-water partition coefficient are responsible for the tendency of B(a)P to partition into the organic fraction of soils with little desorption occurring. The strong attraction of B(a)P to soil limits its mobility through a soil profile making it highly unlikely to be leached to ground water sources

(CCME, 1997). It is the sorption of B(a)P that is thought to explain its limited degradation in sediment (Grosser *et al.*, 1991). The possible degradation pathways for fungi are outlined in Figure 2.5. The sequence of degradation intermediates involved in the bacterial degradation pathway have not been identified at this time (Pothuluri and Cerniglia, 1994).

B(a)P is considered to be the most carcinogenic PAH (Sims and Overcash, 1983) and is considered to be a non-threshold toxicant where any level of exposure is thought possibly to lead to adverse affects (CCME, 1997). It has been found to be involved with chromosomal abberations, DNA adducts, unscheduled DNA synthesis, and sister chromatid exchange (Pothuluri and Cerniglia, 1994). Animal studies have demonstrated that metabolic transformation of B(a)P can result in the formation of a diol epoxide forming in the bay region which once bound to DNA or RNA can lead to the initiation of a tumor (Sims and Overcash, 1983).

While the 4,5-, 7,8-, and 9,10-trans dihydrodiols of B(a)P are rarely metabolized further, the white rot fungi produce 1,6-, 3,6-, and 6,12-quinones that have been shown to fully metabolize to CO_2 (Kanaly *et al.*, 1997). The 4,5- and 9,10-dihydrodiols have a lower carcinogenicity than B(a)P but the 7,8-dihydrodiol is just as carcinogenic as B(a)P itself. The oxide form of 4,5-B(a)P was found to be mutagenic when tested with bacterial and mammalian cells, while the B(a)P 7,8- and the 9,10-oxides had extremely low mutagenic activity (Muncnerova and Augustin, 1994).

Many of the less complex PAH compounds are contained in the structure of B(a)P therefore, it is important to understand how these simpler compounds breakdown. As B(a)P is degraded, it may result in degradation intermediates that resemble naphthalene,

anthracene, pyrene, or other PAHs. If fission of the lone ring on B(a)P were to occur, the resulting four ringed compound may enter the degradation pathway of pyrene (Figure 2.6).

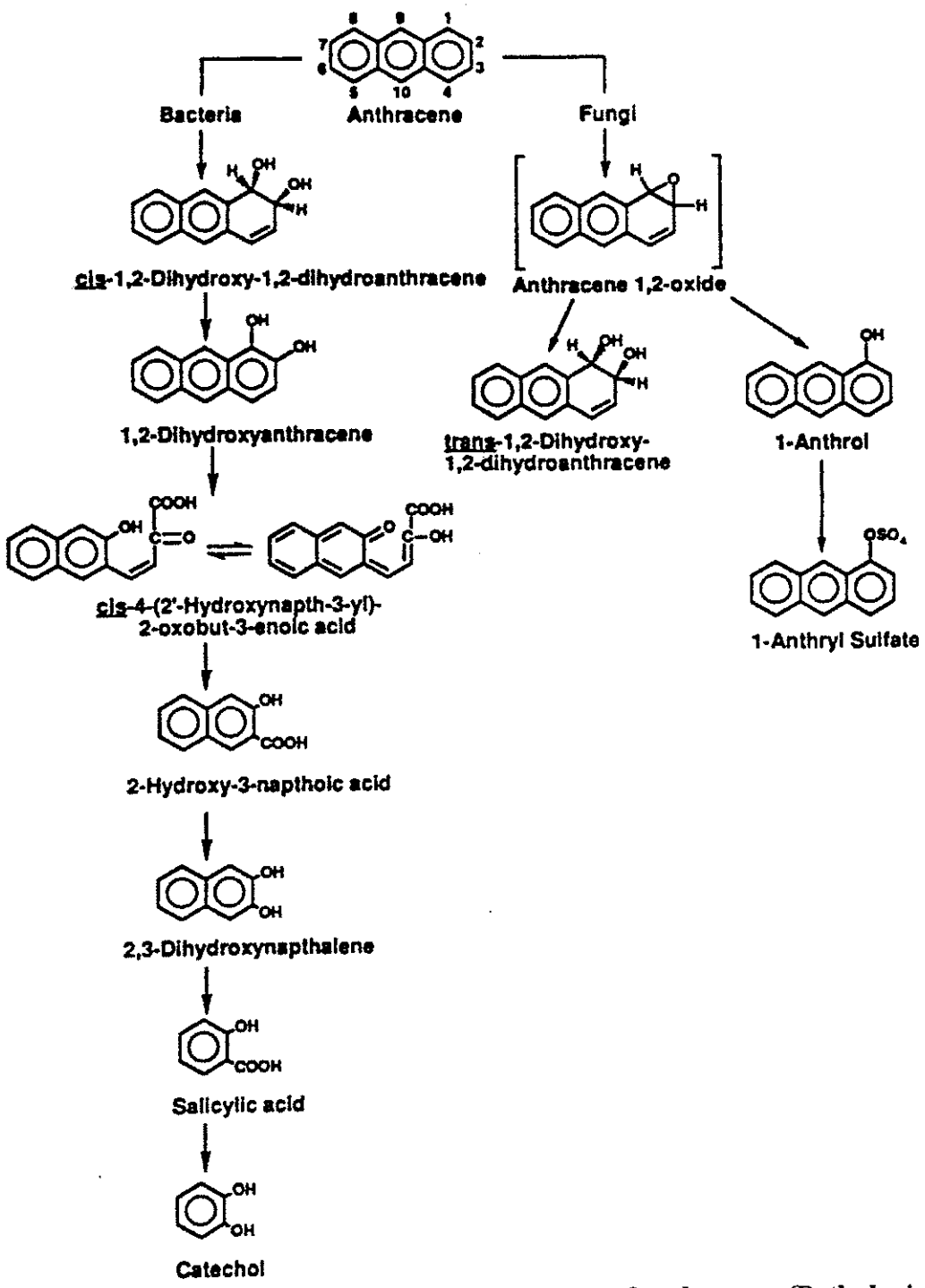


Figure 2.4 Bacterial and fungal degradative pathways of anthracene (Pothuluri and Cerniglia, 1994).

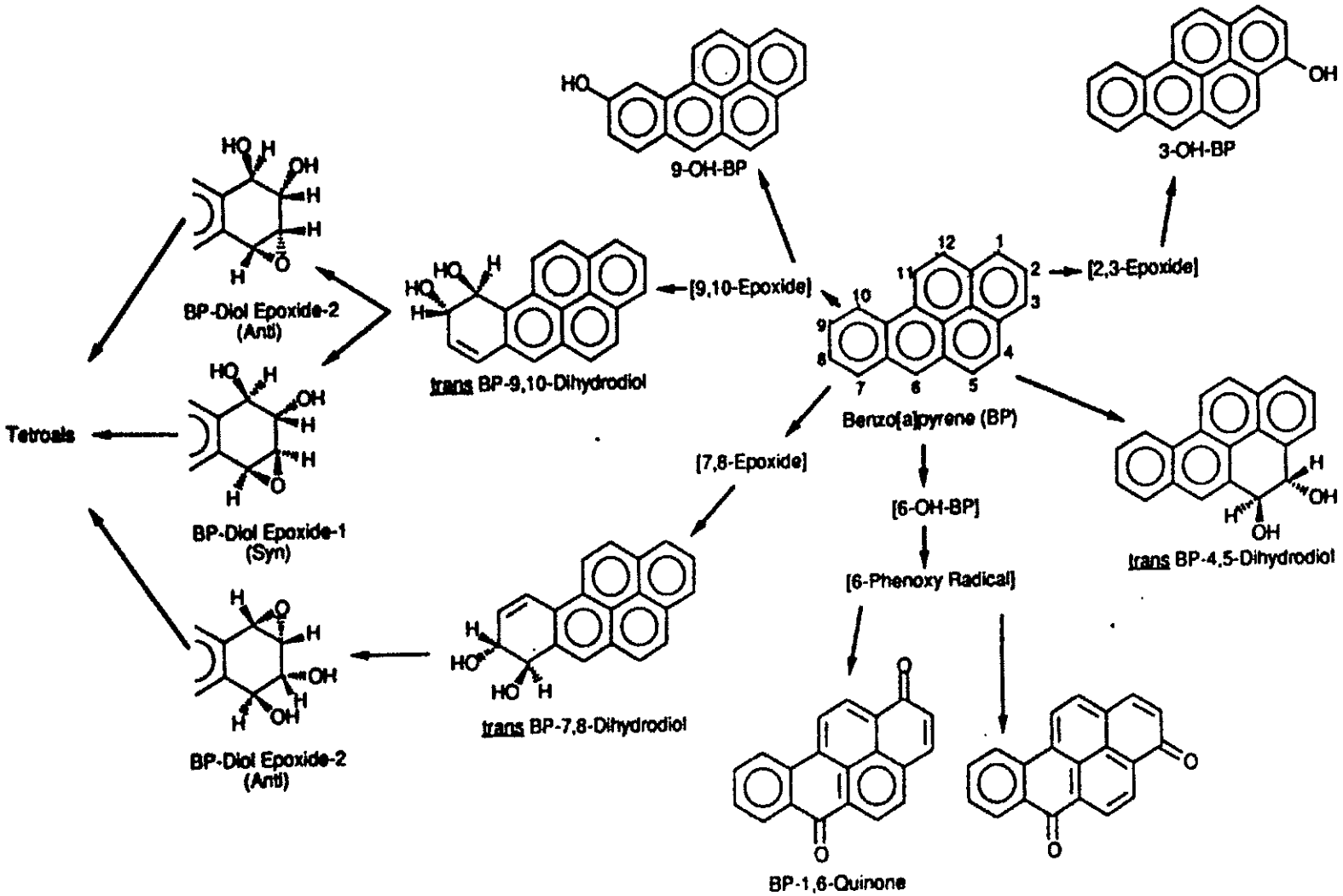


Figure 2.5 Fungal transformation of benzo(a)pyrene (Pothuluri et al., 1994).

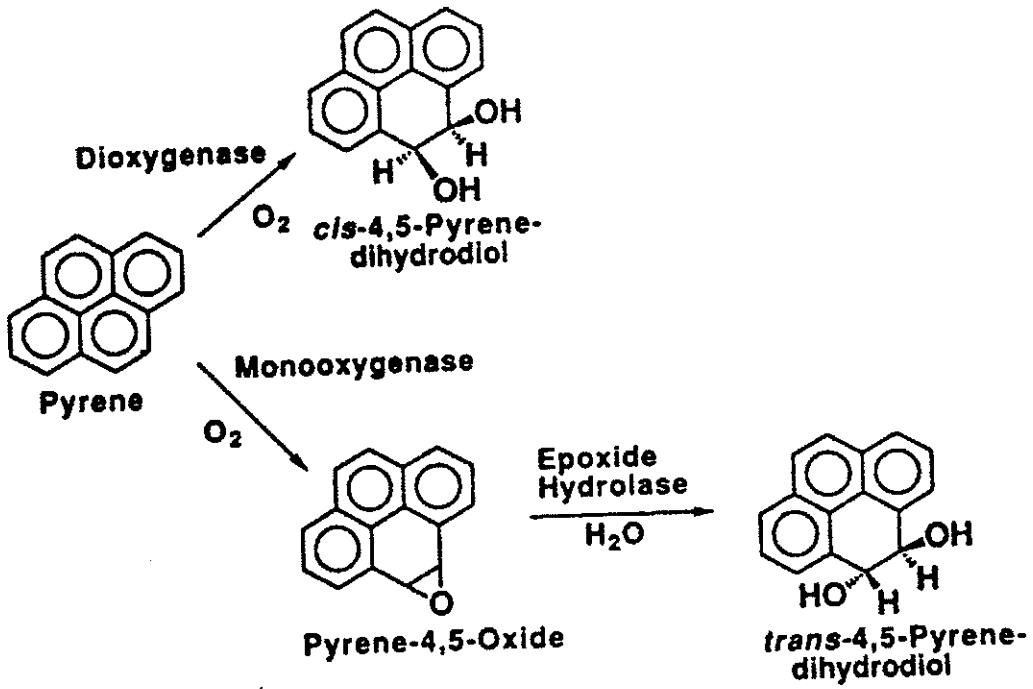


Figure 2.6 Mono- and dioxygenase pathways of pyrene (Pothuluri and Cerniglia, 1994).

2.2 Possible Fates in the Environment

Atmospheric deposition of PAHs produced by industrial processes results in aromatic compounds entering terrestrial systems or entering water. This is one of the principle sources of PAHs entering the environment. Once in an aquatic system PAHs have the tendency to partition into the sediment due to their low solubility and high Kow values. Areas that are close to industrial and residential areas have the highest concentration of PAHs in the soil and sediment (Sims and Overcash, 1983). After an oil spill has resulted in contamination of the environment, the low molecular weight (LMW) compounds may be lost via volatilization, but the high molecular weight (HMW)

compounds remain persistent and their fate depends on the ability of the microbial community to utilize these compounds (Foght *et al.*, 1988). The persistence of many environmental pollutants is thought to result from decreased bioavailability due to the insoluble nature of the compounds or the tendency of the compounds to bind to soil (Aust, 1990; Salanitro *et al.*, 1997). Alternatively, the lack of the required enzymes and the toxicity of some petroleum hydrocarbons to soil microorganisms are believed to be other reasons for their recalcitrance (Salanitro *et al.*, 1997).

2.2.1 Non-Biological Transformations:

PAHs are able to undergo direct photolysis by sorption of solar radiation (Sims and Overcash, 1983), though this process will occur only where surface contamination is exposed to sunlight. Absorption of ultra-violet light by PAHs in the presence of an oxidant such as hydrogen peroxide may transform the parent compound into an intermediate compound that is available for microbial degradation (Wilson and Jones, 1993). Aromatic compounds do not contain groups subject to hydrolysis; therefore, hydrolysis is not a major removal mechanism for PAHs from the environment (Sims and Overcash, 1983).

Volatilization from aqueous solution in the environment is dependent on the characteristic Henry's law constant of the compound and the properties of the environment (wind, temperature, and water turbulence) (Sims and Overcash, 1983). There is an inverse relationship between the number of rings and the loss from soil via volatilization. This removal mechanism becomes negligible for PAHs with greater than two rings (Davis *et al.*, 1993; Sims and Overcash, 1983; Wilson and Jones, 1993).

2.2.2 Sorption to Soil Particles

The movement of a PAH compound through a medium such as soil will be dependent upon the compound itself and the surfaces with which it comes in contact. These hydrophobic compounds tend to adsorb; therefore migrating slowly through the soil profile (Sims and Overcash, 1983), limiting bioavailability (Davis *et al.*, 1993; Field *et al.*, 1995; Wilson and Jones, 1993). The properties of the soil and the compound will determine if the compound is bound by ionic or covalent bonds, or if it is adsorbed by Van der Waals attractions, hydrogen bonding, charge transfer, or hydrophobic bonding (Bollag and Loll, 1983). Adsorption is considered reversible unless covalent bonds have been formed (Bollag, 1992).

Adsorption-desorption kinetics of PAHs are a large factor in determining the bioavailability of these compounds for degradation (Wilson and Jones, 1993). Bioavailability of PAHs in soil may be related to the time period that the contaminants have been present and the tendency for these compounds to sorb to soil organic matter (Shuttleworth and Cerniglia, 1995). In an experiment on the degradation of phenanthrene in soil, a direct relationship between sorption and organic matter content was found that is believed to be responsible for increasing the time requirement for biodegradation (Manilal and Alexander, 1991). The affinity of a chemical for the organic material in the soil is dependent on the organic carbon partition coefficient, K_{oc}, which is a function of the octanol-water partition coefficient, K_{ow}, for that individual compound. PAHs in general have high K_{ow} values and low vapour pressures. This results in their tendency to partition into the soil organic matter (Weissenfels *et al.*, 1992). PAHs have higher K_{oc}

values than other nonpolar compounds, such as chlorinated hydrocarbons, and therefore have an enhanced tendency to partition into soil organic matter (Chiou *et al.*, 1998).

The tendency for a compound to desorb decreases with increasing hydrophobicity so also decreases with an increase in ring number. This indicates a decrease in availability with an increase of complexity of the PAH compound (Smith *et al.*, 1997).

Sorption has two phases. The initial phase is rapid where the hydrophobic adsorbate associates with the hydrophobic surface areas of the soil. This is followed by a second phase that extends over a longer period of time at a much slower rate where the organic compound is transported to the less accessible hydrophobic sites within the soil matrix. Sorption will increase over time until equilibrium is reached where all the hydrophobic sites are occupied by the hydrophobic compound. This suggests that the age of the PAH contamination can affect bioavailability and therefore biotoxicity (Weissenfels *et al.*, 1992). Chung and Alexander (1998) found that the amount of ethanol extractable phenanthrene decreased over a 200-day incubation period. They suggest that the bioavailability of phenanthrene may be limited due to aging of the contamination.

2.2.3 Biodegradation

Soil biodegradation is dependent on the characteristics of each PAH and of the soil environment in which the compound is found (Carmichael and Pfaender, 1997). As the number of rings in a PAH increases the solubility and biodegradation decreases (Grosser *et al.*, 1991; Shuttleworth and Cerniglia, 1995; Sims and Overcash, 1983; Wilson and Jones, 1993). In oil contaminated sediments turnover times of PAHs have been estimated to increase 30 to 100 times per additional ring when going from

naphthalene to benz(a)anthracene. Under certain circumstances two ringed structures like naphthalene may degrade within hours though it may take years for a five ringed structure like B(a)P to turn over (Herbes and Schwall, 1978). In laboratory studies, half-lives have been found to be less than 10 days for two ringed compounds, less than 100 days for three ringed compounds, and greater than 100 days for 4 and 5 ringed compounds (Wilson and Jones, 1993).

The ultimate goal of remediation procedures is to produce less harmful intermediate compounds with the eventual production of carbon dioxide and water. In some cases the intermediate compounds are more toxic, mutagenic, and carcinogenic than the parent compound. Problems can arise if the increased solubility of the intermediate compounds leads to an increased mobility in the environment (Wilson and Jones, 1993). Biodegradation may form more reactive products that result in bound soil residues (Richnow *et al.*, 1995).

Partial oxidation of PAH compounds by white-rot fungi have been shown to transform the compounds to more water soluble compounds with increased bioavailability (Meulenberg *et al.*, 1997). Anthracene, which has a water solubility of approximately 0.07 mg/l, is oxidized by P. chrysosporium to 9,10-anthraquinone and then phthalic acid which have a solubility of 0.6 and 7000 mg/l respectively. It is believed that the initial oxidation of PAHs by the white-rot fungi may increase the bioavailability of the compounds to the indigenous bacteria and, therefore biodegradation is increased (Meulenberg *et al.*, 1997). Carmichael et al. (1997) claim that the initial oxidation of PAHs may be the rate limiting step of PAH degradation. They believe that the reason for lack of detection of PAH intermediate degradation products in soil and sediment is due to

the rapid degradation once the initial oxidation has occurred. The degradation products are more readily available for metabolic breakdown to a larger portion of the microbial community.

2.2.3.1 Mono- and Dioxygenase Metabolic Pathways for PAHs. Incorporation of oxygen into PAH compounds is required prior to enzymatic cleavage of the ring structure. PAH metabolism has been found to include the use of oxidative enzymes such as mono- and dioxygenase. Monooxygenase is produced by fungi and enables a single oxygen addition across a carbon-carbon double bond within the structure of a PAH. Bacteria utilize a dioxygenase enzyme that allows for the incorporation of both oxygen atoms of molecular oxygen into an aromatic compound. Although there is the possibility of monoxygenase forming phenol compounds that are subject to conjugation, both enzymatic routes are thought to produce dihydroxylated structures that further react and result in eventual ring cleavage (Muncnerova and Augustin, 1994; Pothuluri and Cerniglia, 1994; Sims and Overcash, 1983; Wilson and Jones, 1993). The sulphate, glucuronide, glucoside and xyloside conjugates that may be formed during PAH degradation are nonmutagenic compounds (Muncnerova and Augustin, 1994). Figure 2.7 displays the general pathways of the both oxygenase enzymes (Pothuluri and Cerniglia, 1994).

For aromatic ring cleavage to take place, the parent compound initially must be altered by dihydroxylation (Pothuluri and Cerniglia, 1994). Then the microbial communities will transform these metabolites until they become intermediates of a well established degradation pathway, such as those found in the Krebs cycle. Fumaric,

pyruvic, acetic, and succinic acid and acetaldehyde are resulting substrates which are utilized by microorganisms for energy production and protein synthesis (Sims and Overcash, 1983; Wilson and Jones, 1993).

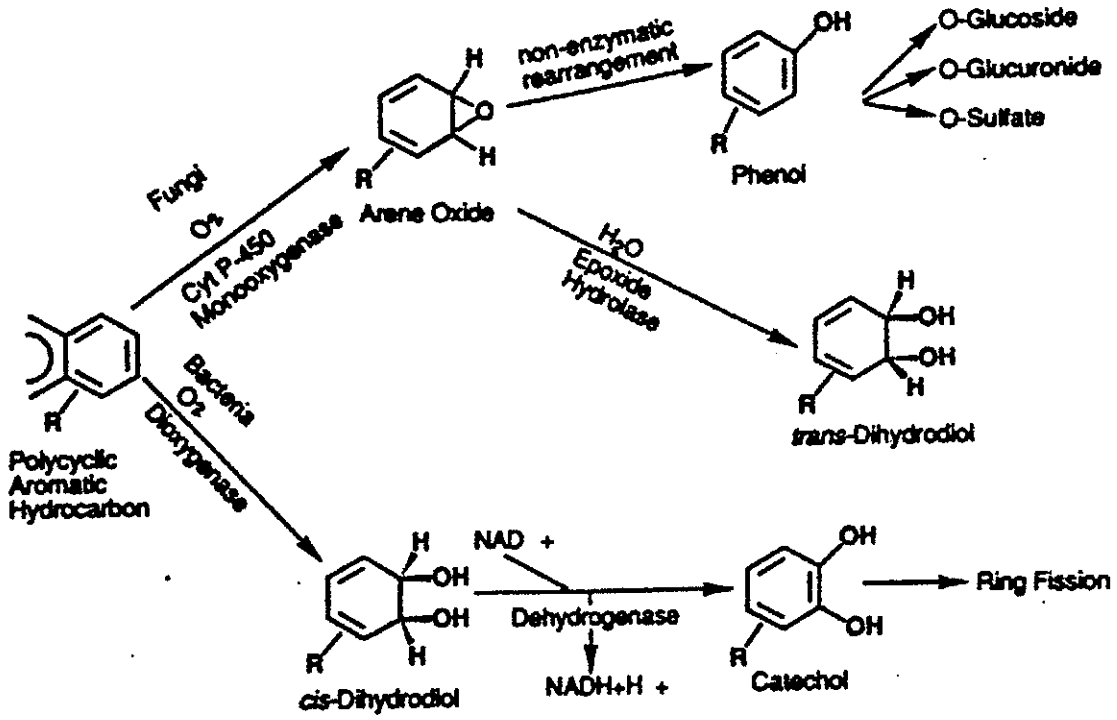


Figure 2.7 Microbial oxidation of PAHs via monooxygenase and dioxygenase pathways (Pothuluri and Cerniglia, 1994).

2.2.3.2 Ligninolytic Degradation of PAHs. Lignin is a complex heteropolymer that functions as a structural component of plant materials (Figure 2.8). It forms a nonrepeating pattern of aromatic, phenol, and biphenol groups joined by carbon-carbon and carbon-ether linkages (Barr and Aust, 1994). The structural irregularity of lignin makes it resistant to the common biochemical cleavage reactions such as hydrolysis

(Hammel, 1992), resulting in lignin being one of the most recalcitrant natural substrates resistant to microbial breakdown (Holroyd and Caunt, 1995).

The lignin degrading process requires nonspecific enzyme mechanisms that enable the breakdown of the many complex structures and the bonding sequences found in lignin. The large size of lignin requires that the initial oxidation steps are extracellular since the transport for intracellular metabolism is not possible (Barr and Aust, 1994; Hammel, 1992). Breakdown of lignin does not occur under anaerobic conditions, since it is an oxidative process that requires oxygen for the initial alteration processes (Kirk, 1984). Ligninolytic activity has a positive correlation with oxygen concentration (Shimada *et al.*, 1981), and has been shown to be inhibited at low oxygen concentration that were high enough to support microbial growth (Kirk, 1984; Kirk, 1990). Ligninolytic activity is not regulated through common reactions of the central carbon catabolism (Fenn *et al.*, 1981) but is suggested to be a process of secondary metabolism regulated by nitrogen availability (Fenn *et al.*, 1981; Fenn and Kirk, 1981; Keyser *et al.*, 1978; Kirk, 1984). The onset of ligninolytic activity is in response to nitrogen depletion and is independent of the presence of lignin itself (Fenn and Kirk, 1981).

Microorganisms degrade lignin because it acts as a physical barrier to an energy rich supply of cellulose and hemicellulose that is contained in the interior of wood materials (Barr and Aust, 1994; Hammel, 1997; Kirk, 1984; Kirk, 1985). Complete lignin degradation is thought to be restricted mainly to a group of microorganisms known as the white-rot fungi (Rodriguez *et al.*, 1996). White rot fungi differ from other microorganisms in their ability to cleave aromatic ring structures (Hammel, 1992; Hammel, 1997).

The extracellular, nonspecific enzymatic system utilized for degrading the complex structure of lignin allows for the breakdown of a wide range of environmental pollutants. The system does not respond to the presence of the substrate itself and does not require a period of conditioning to the presence of the contaminant. The lack of a minimum substrate threshold level required to activate ligninolytic enzymes is thought to allow for low levels of a contaminant to be degraded, as long as other conditions remain suitable (Barr and Aust, 1994). The onset of ligninolytic activity and pollutant degradation in fungal cultures has been found to occur simultaneously and under similar circumstances (Hammel, 1992). It is these similarities that have led researchers to believe that persistent environmental pollutants, such as polycyclic aromatic hydrocarbons (PAHs), can be degraded as a beneficial side reaction of ligninolytic activity (Bumpus *et al.*, 1985; Hammel, 1992; Lamar *et al.*, 1990).

There are structural similarities between PAHs and lignin. PAHs are composed of varying numbers of fused benzene rings and a large portion of lignin is composed of aromatic groups. As in the case with degradation of lignin, high molecular weight PAHs cannot be utilized as a sole source of carbon for microbial growth. An additional primary growth substrate and aerobic conditions are required for the degradation of lignin and PAHs (Hammel, 1992). In many situations PAH microbial breakdown has been found to occur under nitrogen limited conditions (Bumpus, 1989; The National Contaminated Sites Remediation Program, 1993). The National Contaminated Sites Remediation Program has reported that in low level nitrogen environments, there has been successful degradation of five PAHs tested, including B(a)P (The National Contaminated Sites Remediation Program, 1993).

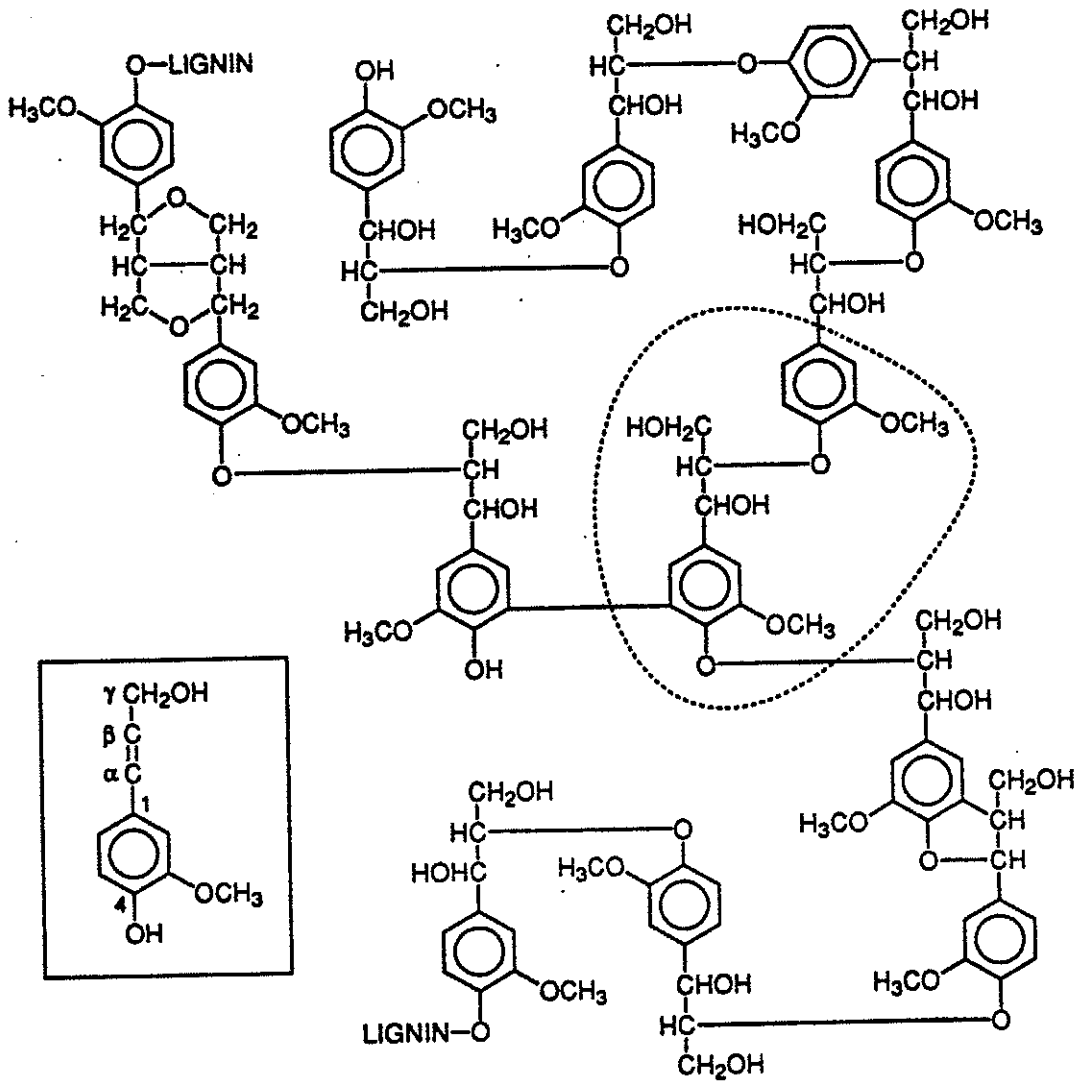


Figure 2.8 Structure of lignin (Hammel, 1997)

2.2.3.2.1 Ligninolytic Enzymes. The two groups of enzymes that function to degrade lignin are laccase and peroxidase (Youn *et al.*, 1995). Lignin peroxidases (LiP), manganese peroxidases (MnP), and laccases also have been linked to the degradation of environmental pollutants (Bogan and Lamar, 1996).

Peroxidase enzymes are known to catalyze polymerization and depolymerization reactions in lignin (Barr and Aust, 1994; Youn *et al.*, 1995). All peroxidase enzymes

contain a iron porphorin ring and require the presence of peroxides for initiation of oxidation activity (Bollag, 1992). White-rot fungi possess the ability not only to produce extracellular peroxidase, but also to convert molecular oxygen to hydrogen peroxide by a second enzyme system (Barr and Aust, 1994).

The lignin peroxidases (LiP), also referred to as ligninase, are the largest group of peroxidase enzymes. The activated states of LiP are considered to have the highest reduction potential of all peroxidase enzymes (Hammel, 1992; Hammel, 1997), but are destroyed by high concentrations of peroxide (Haemmerli *et al.*, 1986). Activation of these enzymes results in carbon-carbon bond cleavage (Hammel, 1992), demethylations, hydroxylations, dimerizations, and benzylic alcohol oxidations (Barr and Aust, 1994). LiP can directly oxidize aromatic compounds by one electron (Hammel, 1992) and this results in the formation of aryl cation radicals. The radicals then have the tendency to undergo spontaneous rearrangement and degradation (Bogan and Lamar, 1995). Alternatively, indirect oxidation can occur where a chemical is oxidized by LiP, then continues to initiate a chain reaction of chemical oxidations (Barr and Aust, 1994). Benzo(a)anthracene, benzo(a)pyrene, and pyrene are examples of three PAHs that have been shown to be oxidized by LiP (Vazquez-Duhalt *et al.*, 1994).

Most white rot fungi produce manganese peroxidases (MnP) which are very similar to other peroxidases. These enzymes are not strongly oxidizing though they are able to oxidize the more reactive compounds like phenols (Hammel, 1997). MnPs contain a Mn^{+2} unit that, when converted to its Mn^{+3} state, is used to oxidize compounds. Hydroquinones are oxidized to semi-quinones that can act as reducing agents (Barr and Aust, 1994). MnP reaches condensed areas of lignin that are inaccessible to other types

of enzyme attack (Hammel, 1992). MnP is also thought to be responsible for oxidizing lignin indirectly via the production of oxyradicals. Unsaturated lipids may undergo peroxidation by MnP in the presence of Mn^{2+}; then these highly reactive compounds oxidize other molecules (Hammel, 1997).

Laccase is a blue copper oxidase that contains four copper ions that form the active site for the catalytic function of the enzyme. It is an enzyme which is responsible for a single electron removal from a phenolic hydroxyl group that leads to the formation of phenoxy radicals. These radicals couple their reactions with other radicals which result in polymerization or depolymerization of lignin model compounds. Polymerization appears to be the dominant of the two types of reactions catalyzed by laccase (Youn *et al.*, 1995).

Phenolic compounds are oxidized to form extremely reactive anionic free radicals. Activation of these enzymes requires the presence of molecular oxygen, but unlike the peroxidase enzymes, laccase does not require hydrogen peroxide to drive the activity (Bollag, 1992). Laccase also possesses an ability to form Mn(II) chelates that have the ability to penetrate the walls of wood cells. Together with LiP and MnP, laccase is thought to be capable of degrading lignin (Youn *et al.*, 1995).

2.2.3.3 Cometabolism. Kanaly et al. (1997) suggest that the degradation of PAHs in soil in the presence of other petroleum hydrocarbons initially may require cometabolic oxidation and then be metabolized further by a consortium of hydrocarbon degrading microorganisms. Researchers have identified microorganisms capable of metabolizing low molecular weight PAHs (two and three rings) as sole carbon sources and energy

sources while degradation of high molecular weight PAHs (four or more rings) most often have been reported to occur due to cometabolism (Bumpus, 1989; Kastner *et al.*, 1994; Pothuluri and Cerniglia, 1994; Wilson and Jones, 1993). Cometabolism occurs when one substrate is used for energy and growth of the microorganisms while the pollutant is concurrently transformed without providing benefits to the microbial community (Glaser and Lamar, 1995). Studies have found that high molecular weight PAHs are more likely to degrade in soil when found in a complex mixture with other hydrocarbon compounds rather than when they are present in the soil as a lone compound (Wilson and Jones, 1993). Wood, annual plant stems, and rice also have been found to be suitable cosubstrates to enhance the degradation of B(a)P (Field *et al.*, 1995). Other studies have demonstrated cometabolism of complex PAHs such as pyrene, B(a)P, benz(a)anthracene, and dibenz(a,h)anthracene when in the presence of the less complex PAHs, naphthalene or phenanthrene (Herbes and Schwall, 1978). The presence of hydrocarbons that are easily biodegradable can supply a carbon source for microbial growth (Lesage *et al.*, 1997).

Bumpus (1989) studied the degradation of ^{14}C-labeled phenanthrene by *Phanerochaete chrysosporium* in a nutrient limited environment. Bumpus found that supplemental glucose addition increased the rate of phenanthrene mineralization. This demonstrates the dependence of the microbial community to utilize an additional carbon source when degrading phenanthrene.

Carmichael et al. (1997) did not find a significant correlation between the bioavailability characteristics of PAH compounds, such as solubility, K_{ow}, soil f_{oc}, and total PAH contamination, with community size or the number of degraders. They suggest

that this is an indication of the cometabolic nature of PAH metabolism where growth and population numbers are dependent on the presence of an additional carbon source.

2.2.3.4 Competitive Metabolism. Research has found a competitive interaction that influences the order to which PAH compounds will degrade. The simpler PAH compounds are degraded before the more complex. It has been suggested that this provides evidence of a shared enzyme pathway that exists to degrade PAHs (Stringfellow and Aitken, 1995). The presence of naphthalene and methylnaphthalenes have been found to interfere with the degradation of phenanthrene (Stringfellow and Aitken, 1995) and other high molecular weight PAHs (Kanaly *et al.*, 1997; Shuttleworth and Cerniglia, 1996). In one case naphthalene has been found to inhibit the growth of bacteria even in the presence of alternative substrates (Shuttleworth and Cerniglia, 1996).

Kanaly et al. (1997) found that when ^{14}C-B(a)P was added to soil in a crude oil mixture, there was a 52 day lag period before the evolution of $^{14}CO_2$ was observed, but after 150 days 50 % of the ^{14}C added to the soil had been converted to $^{14}CO_2$. They suggest that the initial inhibition of B(a)P metabolism may be due to the presence of two ringed PAHs. Foght et al. (1989) refer to a study done by Westlake and Cook in 1980 where they performed a hydrocarbon degradation study. The evolution of $^{14}CO_2$ followed the order of naphthalene, phenanthrene, hexadecane, and anthracene. The simplest PAH was degraded before the more complex PAH compounds.

2.2.4 Humification

Incorporation of PAHs into humic polymers has been suggested as an alternative fate to degradation of these aromatic compounds. Covalent binding of xenobiotics to humic materials results in persistent complexes (Bollag, 1992; Bollag *et al.*, 1988). Plant peroxidases catalyze the polymerization reactions that form lignin. This process utilizes a free radical mechanism similar to the depolymerization system the white rot fungi use to break down lignin (Barr and Aust, 1994). Field et al. (1992) referred to results reported by van der Trench and Sandermann in 1981, where the quinone form of benzo(a)pyrene was found to be incorporated into lignin by horseradish peroxidase. Vascular plants utilize peroxidases and laccases for oxidative coupling of monomers, such as coniferyl alcohol, sinapyl alcohol, and p-hydroxycinnamyl alcohol, to form radicals that will further polymerize to form complex configurations (Hammel, 1997).

In a study by May et al. (1997) the white rot fungus *Phanerochaete chrysosporium* mineralized 2.5 % of the added ^{14}C-B(a)P, yet overall PAH concentration of the soil was reduced by 45 %. The decrease in PAH concentration was thought to be due to polymerization activity

Phenolic compounds and their degradative intermediates resemble naturally occurring humic acids. During humification it is believed that these organic compounds can be incorporated into the humus structure along with the humic acids. Upon humification the compound is thought to be less available to interact with the biota; therefore, decreasing the toxicity of the parent compound, and reducing the possibility of movement via leaching (Bollag, 1992; Bollag *et al.*, 1988; Richnow *et al.*, 1995; Shannon and Bartha, 1988). Soil-bound pollutants are chemically transformed (Muncnerova and

Augustin, 1994) and are thought to resist degradation by becoming less available to the enzymes present in the soil (Davis *et al.*, 1993). The stability of these complexes and the possibility of future release of hazardous pollutants have been questioned. Type of pollutant, soil conditions, microbial activity, and binding mechanisms are factors involved in determining if these bound residues are released, metabolized, or rebound to soil. The release of bound residues is thought to be a gradual process that occurs via microbial activity (Bollag, 1992). Richnow et al. (1995) suggest that the binding of residues in a form that is not extractable with organic solvents to be a long term sink for xenobiotics in the environment (Richnow *et al.*, 1995).

Laccase is thought to decrease the toxicity of pollutants due to the polymerization reactions that render them less available. Its lack of specificity results in its ability to couple pollutants with natural occurring compounds (Bollag *et al.*, 1988). Laccase can become bound to the soil complex itself which results in increased biochemical stability and enhanced ability of the enzyme to catalyze the coupling of substrates (Bollag, 1992).

2.3 Factors Affecting Fate

2.3.1 Aeration

Under anaerobic conditions unsubstituted PAHs are extremely resistant to catabolism by microorganisms (Manilal and Alexander, 1991; Pothuluri and Cerniglia, 1994). Soil moisture conditions have been suggested to be in their optimal range for PAH degradation when maintained between 30 and 90 % of water holding capacity.

Optimal oxygen content has been stated to be 10-40 % (Wilson and Jones, 1993) and oxygen is essential for PAH degradation (Pothuluri and Cerniglia, 1994).

Fields et al. (1995) stated that aeration is the most important factor in successful degradation of PAHs. They found that B(a)P was metabolized to the greatest extent when the aeration status of the soil was artificially enhanced by the addition of pumice stones to the soil. In the presence of a cosubstrate 80 % degradation occurred within a twenty-two day period. It was assumed that the remaining 20 % of B(a)P in the soil sample was resistant to degradation. Though these conditions do not represent an environment that would be encountered at a PAH contaminated site, it does imply that oxygen plays an important role in the degradation of PAHs, as was found to be the case for lignin degradation.

Manilal et al. (1991) examined the effects of soil aeration on phenanthrene breakdown. By increasing aeration of the soil, catabolism of phenanthrene was increased. The effects of increased oxygen were greater than those observed due to an additional supplement of glucose (300mg/kg soil). When phosphorous was added to the soil with increased aeration, the largest rate of degradation was observed.

The importance of oxygen for PAH metabolism was also demonstrated by Hammel et al. (1986). Their experiments suggested that in a culture fluid lignin-peroxidase oxidation of the PAH compound obtained the oxygen from water molecules and not from molecular oxygen. The high level of aeration may have been required to maintain a high redox potential in the culture fluid.

2.3.2 Nutrient Status

Nutrient status of the soil may affect the persistence of PAHs in the environment. Manilal and Alexander (1991) found that phosphorous addition at, 150 mg P/kg soil, increased mineralization of phenanthrene. Doubling the phosphorous to 300 mg P/kg soil did not enhance degradation beyond the level achieved by the addition of 150 mg P/kg soil. Nitrogen addition at 140 mg N/kg soil decreased the amount of phenanthrene metabolized. A combination of nitrogen and phosphorous had a positive effect on the degradation of phenanthrene but the percent mineralized was less than when phosphorous alone was applied to the soil.

P. chrysosporium mineralizes phenanthrene in a nitrogen-limited environment. The rate of degradation leveled off after the initial degradation period. At this time glucose was added and the rate of phenanthrene mineralization increased (Bumpus *et al.*, 1985).

Ligninolytic activity in Bjerkandera sp. strain BOS55 was not inhibited by the presence of nitrogen. When nitrogen levels were below 10 mM NH_4^+-N, the microbial activity was limited and at high nitrogen concentrations the cultures were able to degrade anthracene (Kotterman *et al.*, 1994)

2.3.3 Effect of pH

Different pH ranges have been found to be optimal for PAH degradation . These have been between 7 and 7.8. The pH of a soil will greatly influence the composition of the microbial community. Fungi are more tolerant to acid conditions than bacteria. At low pH fungi have a competitive advantage for growth. If the pH is increased to neutral

conditions, then this provides a more favorable environment for bacteria (Wilson and Jones, 1993). In an acidic soil environment monooxygenase regulated pathways would dominant PAH degradation while in a neutral pH soil environment dioxygenase enzymes would be responsible for the initial oxidation step.

2.3.4 Temperature

Biotic and abiotic losses of PAHs from soil can be influenced by temperature. An increase in temperature affects the chemical properties of PAHs by increasing vapour pressure and solubility while decreasing adsorption to soil colloids. The ability of a microbial community to degrade a compound is related to its solubility and therefore availability (Maliszewska-Kordybach, 1993). The ideal temperatures for PAH metabolism has been reported to be from 20 to 30°C (Wilson and Jones, 1993). A ten degree increase in temperature, 20 to 30°C, increased the rate of degradation of B(a)P by 75% (Field *et al.*, 1995). Coover and Sims found that increasing temperature from 10 to 30°C increased the rate and extent of loss of the low molecular weight PAHs but had little effect on the higher molecular weight five and six ringed PAHs (Coover and Sims, 1987).

2.3.5 Soil Contaminant Concentration and Previous Exposure

Rate of degradation of PAHs is directly proportional to the initial concentration in soil. This indicates that at low concentrations of contaminants the degradation may be occurring very slowly (Aust, 1990; Sims and Overcash, 1983). Field et al. (1995) tested the effect of initial concentration of the rate of B(a)P degradation. B(a)P in acetone was

introduced to unpolluted soil at 100 and 1000 mg/kg. The rate of initial degradation doubled with an increase in concentration by an order of magnitude. Sims et al. (1983) state that degradation of B(a)P can occur under natural conditions though breakdown is a function of soil concentration of B(a)P and previous exposure to PAHs. Non-acclimated soils showed low rates of B(a)P breakdown if it was demonstrated at all. In soils with significant levels of PAH residue Kastner et al (1994) found bacteria that were able to degrade PAHs more complex than naphthalene. They suggest that PAH degrading bacteria may be present in a soil but a minimum concentration of PAHs may be required to stimulate growth of the PAH degrading microorganisms. At this time a threshold concentration of PAH contamination that is required to activate PAH degrading organisms is not known.

Soils that have been exposed to hydrocarbon contaminants have been found to degrade PAH compounds at a higher rate than in unacclimatized soils (Wilson and Jones, 1993). B(a)P was found to be degraded at a faster rate by bacteria isolated from soil containing B(a)P than bacteria that were isolated from uncontaminated soil (Sims and Overcash, 1983). Bauer et al. (1988) found that preexposure to PAH compounds increased the mineralization of naphthalene.

The type of contamination influences the successfulness of the adapted microbial community to degrade PAHs. Soils with diesel-oil-contamination have displayed an increased ability to degrade PAHs to low levels in short periods of time. Wood treatment activities have resulted in sites with a high concentration of PAH contamination and remediation efforts have had limited success at these locations (Wilson and Jones, 1993). Microorganisms that degrade PAHs also may need to tolerate the presence of other toxic

compounds such as cyanide or PCP found at contaminated sites (Baker Lee *et al.*, 1995; Weissenfels *et al.*, 1992). PCP is a potent fungicide that was used as one of the components in wood treatment (Glaser and Lamar, 1995) that has been found to be degraded by P. chrysosporium. The rate of degradation was found to be related to growth and metabolic activity which are influenced by the nutrient status of the soil (Lamar *et al.*, 1990).

2.3.6 Ionization Potential

The ability of LiP to oxidize PAHs was initially reported by Hammel et al (1986). They established a clear correlation between ionization potential and the suitability of certain PAHs compounds to be utilized as LiP substrate. It was determined that lignin related compounds undergo oxidation catalyzed by LiP as long as the compound has an ionization potential of less than or equal to 7.55 eV. Ionization potential was defined as the amount of energy that was required to remove the first electron from the PAH compound in order to form its cation radical (Table 2.3).

Horseradish peroxidase has been reported to have similar results for binding PAH to DNA via cation radicals, but can only oxidize compounds with ionization potentials as high as 7.35 eV. Consequently the hydrogen peroxide activated states of the LiP enzymes are more electropositive and therefore have the ability to oxidize compounds with higher potentials. It was concluded that the one-electron oxidation mechanism of LiP used to oxidize lignin was also used to oxidize certain PAH compounds and other heteroaromatic pollutants (Hammel *et al.*, 1986).

Bumpus (1989) found that a nitrogen limited culture of *Phanerochaete chrysosporium* was able to degrade phenanthrene which has an ionization potential of approximately 8.0 eV. Oxidation of this compound was explained by the assumption that there must be LiP enzymes with even higher ionization potentials than that reported by Hammel et al. (1986).

Bogan and Lamar (1995) suggest that MnP plays a key role in oxidation of PAHs. In a study that used manganic acetate, it was found that PAHs with ionization potentials equal to or less than that of chrysene (7.8 eV) could be oxidized by Mn^{3+}. It is suggested that it may be MnP that is responsible for the oxidation of PAHs with high ionization potentials, such as fluoranthene or phenanthrene, though at this time the role of MnP in degrading these compounds is still unclear.

Twelve PAHs that ranged from 3 to 6 ringed compounds were found to have percent recoveries that correlated to their ionization potential when incubated in liquid cultures studies with *P. chrysosporium*. The inverse relationship between ionization potential and degradation is evident; therefore, compounds with lower ionization potentials should be more readily oxidized. Oxidation of PAHs with ionization potentials between 7.2 and 8.1 eV was observed, indicating that a one-electron oxidant with a higher ionization potential than LiP and MnP must be involved (Bogan and Lamar, 1995).

The high level of phenanthrene degradation deviates from the trend and is thought to be explained by the increased level of solubility of this PAH in relation to the other compounds used in the experiment. Anthracene, pyrene, B(a)P, and benz(a)anthracene have lower recovery rates than would be expected from their ionization potentials. All four of these compounds have very low half-lives in the gas-phase reaction with hydroxyl

ions. This appears to be related to the susceptibility of these compounds to form oxy radical addition reactions. This mechanism would contribute to the observed degradation of these compounds, leading to a greater than predicted disappearance (Bogan and Lamar, 1995).

Ionization potential of the individual PAH compound appears to be an important factor in determining the resistance of these pollutants to degradation by ligninolytic enzymes. The threshold potential determined by Hammel et al (1986) seems to either underestimate the oxidation capacity of LiP or may have been specific for that type of LiP enzyme tested during this experiment. It appears that further investigation is needed in order to acquire a complete understanding of the effect the ionization potential of lignin degrading enzymes has on the metabolic breakdown of organic pollutants in the environment.

CHAPTER 3

Degradation of Benzo(a)pyrene in Soils from Four Catena Sites

3.1 Abstract

Benzo(a)pyrene [B(a)P] is a polycyclic aromatic hydrocarbon (PAH) that is carcinogenic in nature. The purpose of this study was to examine the influence of landscape position and soil type on the ability of the indigenous microbial community to degrade B(a)P. Landscape position influences the nature of soil and its metabolic capacity. Sites were chosen that are representative of an association of soil types which occur in a sloping landscape known as a catena. Microcosms were used to contain the soil samples to which ^{14}C-labeled B(a)P in a diesel fuel stock solution was added. Degradation and volatilization were monitored for the duration of the experiment.

The level of $^{14}CO_2$ evolution did not reach significant levels from any of the soils used in this experiment. Drying and wetting the soil, amending the soil with straw, and inoculating the soil did not increase B(a)P degradation. The low levels of degradation observed in this experiment may have resulted due to the limited bioavailability of this compound, although in this experiment we have not accounted for any transformation of B(a)P that may have been incorporated into the soil or the microbial biomass.

3.2 Introduction

PAHs have become widespread environmental pollutants. These compounds form a family of benzene derivatives, composed of varying numbers of fused aromatic rings that range in complexity from simple two ringed structures, like naphthalene, to condensed multi-ringed substances, such as B(a)P. In the environment PAHs create potential health problems and remain persistent throughout ecosystems (Pothuluri and Cerniglia, 1994).

Incomplete combustion of organic-carbon based material during industrial processing such as petroleum refining, coal gasification and coal liquification are responsible for the generation of PAHs (Miller *et al.*, 1988). The increased level of PAHs in the environment has been largely a result of the burning of fossil fuels over the last century. The toxic, mutagenic, and carcinogenic properties that some of these compounds possess have created great concern (Pothuluri and Cerniglia, 1994) and have lead to the addition of various PAHs to the Priority Substances List of the Canadian Environmental Protection Act (CCME, 1991). One of these pollutants is B(a)P, a 5 ringed PAH that is a known carcinogen. Mammalian systems metabolically activate B(a)P into DNA-binding products that lead to tumor formation in animal testing (Phillips, 1983).

The number of rings in the structure of a particular PAH is directly related to the stability of that compound. High molecular weight PAHs, containing 4 or 5 rings, have low water solubility and remain extremely recalcitrant once introduced into the environment (Grosser *et al.*, 1991). It is the hydrophobicity of these

compounds that is thought to be responsible for their persistence in soils (Schnitzer and Schuppli, 1988). Turnover times for PAHs have been found to increase 30 to 100 times per additional ring (Herbes and Schwall, 1978). Once introduced into the environment, the behavior of the contaminant will be dependent on the type of PAH present. PAHs with low molecular weight may volatilize and be lost to the atmosphere, while the larger compounds will remain behind. The persistence of these pollutants in the environment is dependent on the ability of the microbial population present in the ecosystem to metabolize these substances (Foght *et al.*, 1988).

Landscape position influences the nature of the soil microbial environment and its metabolic capacity. Sites were chosen for this study that are representative of an association of soil types which occur in a sloping landscape known as a catena.

Hydrology of a site is a major factor in determining the characteristics of the microenvironments that exist within a landscape. The moisture content over a catena will increase as you move from the crest to the toe. This is due to run-off from higher locations and run-on to lower positions, as well as changes in elevation relative to the water table. Since the known degradation pathways of PAHs are dependent on the presence of oxygen, the soil moisture content will influence the processes that are able to occur throughout the landscape and that contribute to the occurrence and persistence of these contaminants within the environment.

The four soil associations selected for this study have been subjected to different climatic conditions during their development. This is reflected in the

properties of the soils. The organic matter content of soil varies within a catena and between sites due to the processes that can occur at each location. Since organic matter content forms a direct relationship with sorption of PAHs (Weissenfels *et al.*, 1992), it is also an important component in determining the bioavailability of these contaminants for microbial degradation.

3.3 Objective of the Study

The objective of this study was to examine the influence of landscape position and soil type on the ability of the indigenous microbial community to degrade a persistent contaminant such as benzo(a)pyrene. Soil samples taken along a catena provided a range of microenvironments that were influenced by the local hydrology and soil properties of each individual site.

3.4 Materials and Methods

3.4.1 Site Description

Four catena sites were selected for this study: Thin Black (Ryerson Association SE 22-4-29 W), Black (Newdale Association NE 31-12-18 W), Dark Gray (Erickson Association NW 14-19-23 W), and Gray Luvisol (Waitville Association SE 4-20-23 W). These soils are representative of a transition from the semi-arid prairie region (Thin Black) to the boreal Parkland area (Gray Luvisol). Soil samples were taken from four positions along the sloping landscape that is

typically found in a prairie pothole region: crest, upper mid-slope, lower mid-slope, and toe (Table 3.1). Each catena site was established on a south-facing slope that formed a range of moisture regimes from a well-drained crest to a poorly drained depressional zone (Burton, 1995).

Soil properties differ between sites as well as with position of the catenas (Table 3.2). The level of organic matter varied over a short distance, increasing from the crest to the toe of a catena. Sorption by organic matter and soil particles will differ with site position and soil type. Water holding capacity is greatly influenced by soil organic matter, which in turn determines the aeration of the soil environment. For a more detailed soil description of these four sites refer to appendix II a to II d.

Differences in pedogenic processes are due in part to differences in hydrology. The soil properties at a site influence the composition of the microbial community. A soil at the crest position experiences a greater amount of water run-off and does not receive run-on from the surrounding area. Soils in this portion of the landscape experience extended periods of water stress. Mid-slope positions receive run-on from the up-slope positions but also experience run-off to the lower toe positions. The toe locations have extended periods of high water content and reduced oxygen availability occurs. This is evident by the mottling observed in the lower horizons of the soils sampled at the toe position from the Ryerson, Waitville, and Newdale associations. Local hydrology results in a reallocation of moisture from upper to lower hillslope positions and may be accountable for differences in soil characteristics over very short distances. These differences can influence the

microbial community and metabolic capacity that exist at each location, therefore determining the potential for xenobiotiotic degradation.

Table 3.1 Soil description and classification of the catena soils.

Soil	Association	Soil Series	Class
Dark Gray	Erickson	Erickson	Orthic Dark Gray
		Erickson	Orthic Dark Gray
		Varcoe	Gleyed Rego Black
		Drokan	Rego Humic Gleysol
Gray Luvisol	Waitville	Waitville	Orthic Gray Luvisol
		Waitville	Orthic Gray Luvisol
		Petlura	Gleyed Dark Gray Luvisol
		Sinnott	Rego Humic Gleysol
Black	Newdale	Rufford	RegoBlack
		Rufford	Rego Black
		Newdale	Orthic Black
		Varcoe	Gleyed Rego Black
Thin Black	Ryerson	Hathaway	Rego Black
		Ryerson	Orthic Black
		Regent	Gleyed Black
		Tilston	Humic Luvic Gleysol

Table 3.2 Soil properties of the top 10 cm of the soils from the four catena sites.

Soil	pH	Electrical Conductivity (dS/m)	Soil Moisture (%)	Field Capacity (%)	Organic Carbon	Sand (%)	Silt (%)	Clay (%)	Textural Class
Erickson Crest	7.6	0.26	18.1	24.2					
Erickson Upper Mid-Slope	7.5	0.24	17.7	24.0	2.5	43.5	29	27.5	L
Erickson Lower Mid-Slope	7.8	1.90	27.4	25.2					
Erickson Toe	7.8	2.23	29.7	29.3					
Waitville Crest	7.4	0.25	21.2	25.4					
Waitville Upper Mid-Slope	7.7	0.81	30.3	25.1	1.1	34.5	33.5	32	CL
Waitville Lower Mid-Slope	7.6	0.35	22.5	27.6					
Waitville Toe	6.8	1.72	66.5	52.4					
Newdale Crest	7.4	0.45	23.5	32.8					
Newdale Upper Mid-Slope	7.3	0.41	24.1	29.0	5.7	28.5	33.5	38	CL
Newdale Lower Mid-Slope	7.4	0.53	27.6	27.4					
Newdale Toe	7.4	1.00	38.0	31.7	4.7	31.5	35.5	33	CL
Ryerson Crest	7.6	0.30	16.2	30.9					
Ryerson Upper Mid-Slope	7.4	0.30	17.1	32.3	3.2	45	28.5	26.5	L
Ryerson Lower Mid-Slope	7.3	0.26	19.4	32.9					
Ryerson Toe	6.7	0.28	22.5	39.7					

3.4.2 Soil Sampling and Preparation

The soil samples were collected in August of 1995. Each catena site was sampled with three replications at the four positions mentioned. Twelve samples were taken per site for a total of 48 plots. A Dutch augur was used to obtain samples of the top 10 cm of soil. For each of the 48 plots, ten augured samples were taken systematically and composited to obtain a representative soil sample. All soils were air dried, sieved (2 mm), and stored until needed.

Twenty grams of each soil on an air dry basis was weighed into a 50 mL beaker and wet to 80 % field capacity. All samples were covered with parafilm and were incubated at 20°C for two weeks. At the end of this period the soil samples were weighed and if needed were rewet to 80 % field capacity. 10 months into the experiment the soils were weighed and rewet to 80 % field capacity. Field capacity values are listed in Table 3.2.

3.4.3 Microcosm Apparatus

Microcosm jars were prepared by gluing a 3 cm piece of polyvinyl chloride (PVC) tubing in the bottom of a 500 mL glass jar. To these jars was added a 50 mL beaker containing the soil sample, a 20 mL vial with 1 mL of acidified water to help maintain humidity, a foam plug to trap any labeled compound that might volatilize during the experiment period (EPA CFR §796.3400), and a 7 mL scintillation vial that contained a carbon dioxide trapping solution (Figure 3.1). The PVC tubing was used to hold the carbon dioxide trap in place.

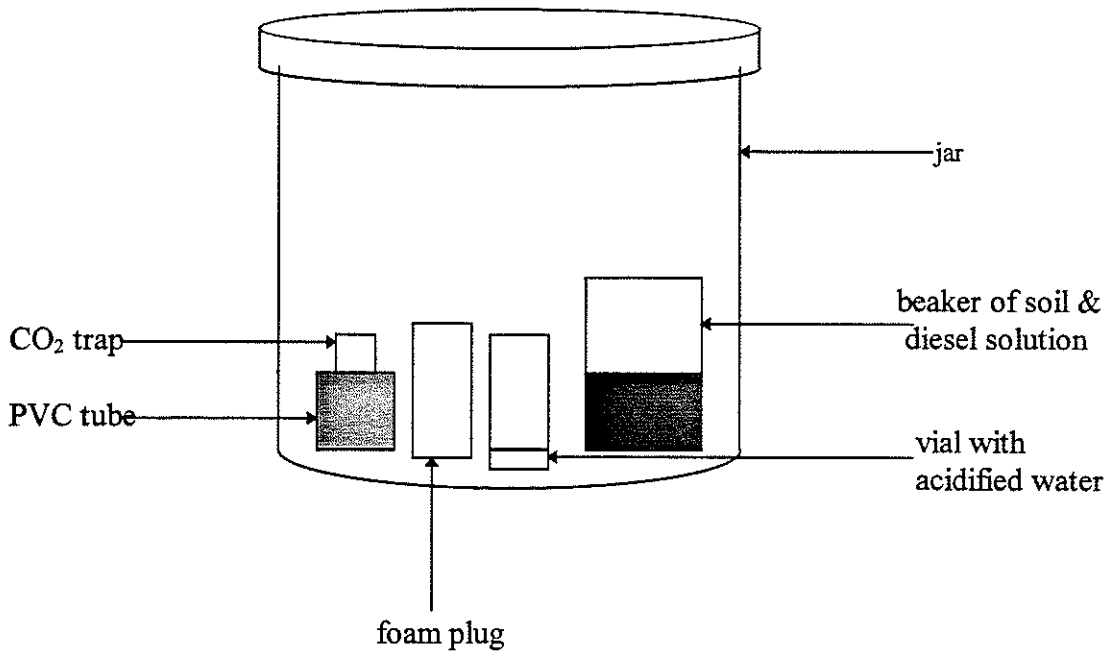


Figure 3.1 Microcosm apparatus used for benzo(a)pyrene degradation study.

3.4.4 PAH Compound Addition

Benzo(a)pyrene-7,10-^{14}C (2.1×10^9 Bq/mmol) was purchased from Sigma chemical company (Figure 3.2). The 7 and 10 position on the B(a)P compound, where the ^{14}C-labels are located, are the two sites initially oxidized in the known degradation pathways (Miller *et al.*, 1988; Pothuluri and Cerniglia, 1994). In order to add B(a)P to our soil samples it was initially mixed with diesel fuel at a concentration of 15 Bq/mg and then the diesel fuel was added to the soil at 5000 μg/g. The total volume of stock solution added to the surface of each beaker of soil was 0.1 mL which resulted in approximately 0.08 μCi per soil sample.

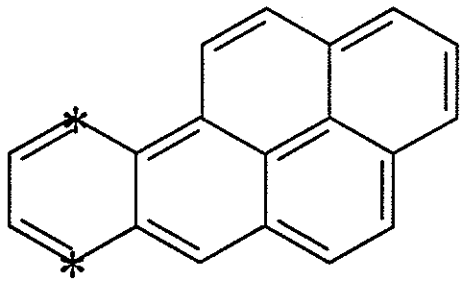


Figure 3.2 Structural formula for benzo(a)pyrene. The "*" represents the location of the ^{14}C-label.

Approximately 16 months after the experiment had begun there was no signs of significant degradation of ^{14}C-labeled B(a)P from any of the 48 soils. It was thought that perhaps a nutrient limitation was responsible for the lack of metabolic breakdown of the contaminant. It was decided that the soil samples should be dried and rewet to field capacity. By drying down the soils a portion of the nutrients held in the microbial biomass would be released to provide additional substrate for the surviving organisms (Kieft *et al.*, 1987; Sparling *et al.*, 1985). Kieft et al. (1987) found that a large portion of the available biomass-C in soil was released after rapid wetting. Rewetting the soils results in favorable conditions for the microbial population to utilize the newly released nutrients and a flush of microbial activity would be expected.

If the wetting and drying treatment did not result in the metabolic breakdown of the contaminant then an additional carbon source was to be added to the soil to induce cometabolic breakdown of B(a)P. Morgan et al. (1993) found that the addition of wheat straw to soil inoculated with white-rot fungi increased the degradation of xenobiotics. Available carbon could have been limiting the overall activity of the microbial community and therefore was suppressing the breakdown of B(a)P. Since straw is readily

available at a minimal cost it is a practical carbon amendment for large scale bioremediation purposes.

3.4.5 Wetting and Drying

The vial of acidified water was removed from each microcosm and was replaced with a 20 mL scintillation vial approximately half full of Drierite. The Drierite previously had been placed in an oven at 110 °C for 24 hours and cooled in a desiccator to ensure that all water had been removed. The vials of Drierite remained in the microcosms for three weeks to allow for the soils to dry down. After this time the beakers of soil were temporarily removed from the microcosms to rewet the soils to field capacity. All samples were placed back in the incubator and $^{14}CO_2$ evolution continued to be monitored on a biweekly basis.

3.4.6 Straw Addition

Wheat straw (< 2 mm) was added to the soil as a carbon amendment one month after the soils had been wet to field capacity. The straw had a carbon to nitrogen ratio of 43.5 to 1. It was weighed into a beaker at a 1:20 ratio of straw to soil (w/w). The soil from the microcosms was then transferred from the 50 mL beakers into the beaker with the straw. They were mixed together, returned to the 50 mL l beaker, wet up to field capacity, and placed back in the microcosms. It was believed that an additional carbon source may have been required to supply the energy necessary for population growth and maintenance while B(a)P was degrading.

3.4.7 Preparation of Inoculum

An inoculum was prepared from a soil from a previous experiment (chapter 4: the industrial soil with a low level of contamination) that exhibited high levels of B(a)P degradation and was added to the catena soils from this experiment. The hypothesis was that the indigenous microbial population of these soils did not possess the capability to perform the initial oxidation steps necessary for the breakdown of this recalcitrant compound and that the addition of the inoculum would initiate degradation of B(a)P.

A 10 g soil sample (on an oven dry basis) that had displayed B(a)P degradation was transfer to a dilution bottle containing 90 mL of saline-agar solution and glass beads. The bottle was placed on a lateral shaker for half an hour. A 1 mL aliquot of solution was used to inoculate the catena soil sample from each microcosm. Following the soil solution addition the beaker of soil was weighed, wet to field capacity, and returned to the microcosm. The evolution of $^{14}CO_2$ was monitored.

The soil sample used to create the inoculum contained ^{14}C-labeled B(a)P from a previous experiment. The level of radioactivity associated with a 1 mL sample of the soil solution was determined by use of a liquid scintillation counter and was taken into consideration during further calculations.

3.4.8 Monitoring $^{14}CO_2$ Evolution

The 7 mL vial contained 1 mL of (+/-)phenylethylamine in methanol (1:1 volume) to trap any $^{14}CO_2$ that evolved during the experiment. For each of the soil samples the sealed jars created an enclosed environment that allowed the labeled compound to be confined within this system. The vials containing the carbon

dioxide trapping solution were added to the microcosms at the same time as the B(a)P and diesel solution was added to the soil. All microcosms were then incubated at 20°C. The carbon dioxide traps were initially changed at 24 hours, 96 hours, and then weekly from the day the stock solution was added to the soil samples. The frequency of the trap changes were dependent on the level of radioactivity determined in the previous set of traps. The total duration of the experiment was 24 months. During this time the microcosms remained in incubation at 20°C.

3.4.9 Liquid Scintillation Counter

After traps were removed from the microcosms, 5 mL of Ecolite scintillation cocktail (ICN Biochemicals Inc. Aurora, OH) were added directly to each vial containing 1 mL of phenylethylamine in methanol. The vials were allowed to sit for a minimum of 24 hours before the level of radioactivity was determined by a Beckman LS 7500 scintillation counter. The level of activity per trap was given in disintegrations per minute (DPM). These values were then used to calculate the percentage of initial radioactive ^{14}C-B(a)P added to the soil that had evolved as $^{14}CO_2$ during the incubation period.

3.4.10 Methanol Extraction of Foam Plugs

Approximately 10 months into the experiment the polyurethane foam plugs were removed from the microcosms and new ones were put in their place. The plugs that had been removed were placed in 100 mL French square bottles (EPA §796.3400). Twenty milliliters of methanol was added to each bottle. The bottles were sealed with rubber stoppers and were placed in a lateral shaker on low for two minutes. It appeared that the foam plugs had adsorbed some of the methanol from the carbon dioxide traps during the incubation procedure. The plugs had begun to crumble when removed from the microcosms and essentially disintegrate during the shaking portion of the methanol extraction process. A 1 mL sample of the methanol extract was removed from each bottle, placed in a 7 mL scintillation vial with 5 mL of EcoLite scintillation cocktail, allowed to sit for 24 hours, and was counted by the liquid scintillation counter. The amount of radioactivity trapped in the foam plugs was determined as a percentage of the radioactivity initially added.

This procedure was repeated at 17 months and again once the experiment had ended. The amount of radioactivity that was lost due to volatilization during the entire experiment was determined by the sum of the results from all three methanol extraction procedures.

3.5 Results

Over the 750 days of this experiment the soils from the four slope positions of the catena at the Ryerson association and the upper two positions of the catena at the Newdale association were the only ones that produced $^{14}CO_2$. The other soils from the catenas located in the Erickson and Waitville associations as well as the lower two positions from the Newdale association had less than 0.2 % of the ^{14}C added evolve as $^{14}CO_2$ during the experiment (Figure 3.3).

During the development of the catena soils the climate and the soil conditions acted as selective pressures that influenced the composition of the microbial community found at each sampling location. Elevation differences in a landscape result in differences in aeration status of the soil and in water content fluctuation. Aeration of the soils at the catena sites will decrease from the crest to the toe, which affects the aerobic and anaerobic character of the microbial environment. The water content of the crest position will fluctuate more than in the lower slope positions. The fluctuation in soil moisture content that occurs under field conditions was not reflected in the microcosm apparatus but the properties of the soil itself will have selected for the microbial community that is present.

With the exception of the Newdale association, field capacity values increase from the crest position to toe of the catena. Due to the low water solubility of oxygen the amount of oxygen available to the microorganisms would be expected to dramatically decrease with an increase in moisture content of a soil. Degradation of B(a)P has a positive correlation with soil aeration as indicated by increased

metabolism in the crest and upper mid-slope positions in the Newdale and Ryerson soils. These findings are consistent with previous research since the known degradation pathways for PAHs require aerobic environments to induce the degradation process by incorporating oxygen into the aromatic structure (Pothuluri and Cerniglia, 1994).

Organic matter content of a soil may also be a factor in determining the availability of B(a)P for degradation. Soil moisture content increases when moving from the crest to the toe. Sites with lower elevation have more moisture available to support plant growth and less aeration to increase decomposition, resulting in soils with higher organic matter content (Table 3.2). Since B(a)P sorption and organic matter content form a direct relationship (Weissenfels *et al.*, 1992), sites with increased organic matter may have a larger portion of the PAH unavailable to the microbial population for degradation. High levels of organic matter in a soil and B(a)P's hydrophobic nature may be responsible for its persistence in the environment.

All four sites from the Ryerson association had measurable amounts of $^{14}CO_2$ evolution and this was the site with the lowest level of organic matter content. Although there appears to be an inverse relationship at this site between the organic matter content and B(a)P degradation when moving from the crest to the toe of the Ryerson catena, the overall level of degradation found in this experiment is less than one percent.

290 days after the experiment started, water was added to the soils so that they were at 80% field capacity. The trap change following this addition of water indicated that the soils from the upper two positions from the Newdale catena experienced a slight increase in the evolution of $^{14}CO_2$. At 480 days the soils were all dried down and 20 days

later they were all wet to field capacity. This time the drying/wetting effect did not cause a flush of activity that increased the metabolism of the B(a)P contaminant. The trap changes following the straw carbon amendment indicate only a slightly elevated level of $^{14}CO_2$ in the soils from the upper two positions of the Newdale catena.

Volatilization was not a major loss pathway for B(a)P from the soil. Methanol extraction of the foam plugs found less than 2% of the added radioactivity was lost as volatiles (Table 3.3).

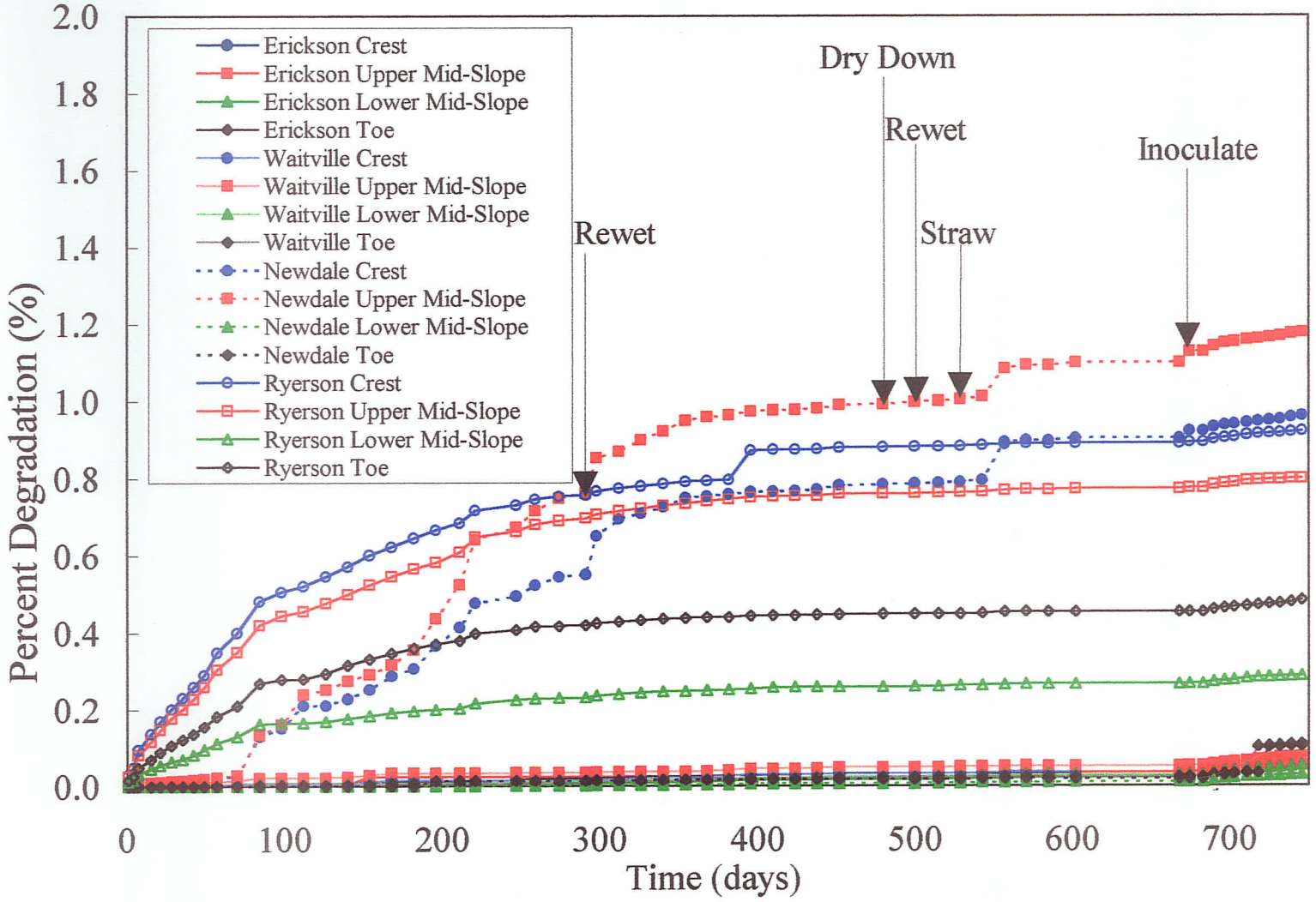


Figure 3.3 Benzo(a)pyrene degradation in four catena soils at the four slope positions. Calculated as the cumulative percent of added radioactivity recovered as $^{14}CO_2$. Points represent the mean of three replicates.

Table 3.3 Methanol extraction of polyurethane foam plugs as a measure of volatile losses of benzo(a)pyrene from the soil. Value in table is the mean of three replications.

Site	Methanol Extract of PUF (%)
Erickson Crest	0.00
Erickson Upper Mid-Slope	0.00
Erickson Lower Mid-Slope	0.03
Erickson Toe	0.00
Waitville Crest	0.03
Waitville Upper Mid-Slope	0.03
Waitville Lower Mid-Slope	0.00
Waitville Toe	0.01
Newdale Crest	0.06
Newdale Upper Mid-Slope	0.05
Newdale Lower Mid-Slope	0.00
Newdale Toe	0.00
Ryerson Crest	0.02
Ryerson Upper Mid-Slope	0.01
Ryerson Lower Mid-Slope	0.24
Ryerson Toe	0.45

3.6 DISCUSSION

This study utilized a range of soil types from different landscape positions to examine the ability of the indigenous microbial community to degrade B(a)P. The Ryerson soils showed the highest level of $^{14}CO_2$ production and the Newdale site showed the beginning of B(a)P degradation in the upper two locations of the catena. There were no signs of $^{14}CO_2$ evolution in the other two sites. In both sites where $^{14}CO_2$ was produced, the highest level of metabolic breakdown occurred in the soils at the higher elevations, the crest and upper mid-slope.

The level of $^{14}CO_2$ evolution did not reach significant levels from any of the soils used in this experiment. The level of purity of the ^{14}C-B(a)P used in this experiment is greater than 98.2% and in all cases the level of B(a)P degradation observed from the catena soils was under 2%. It cannot be determined if these low levels of $^{14}CO_2$ evolution resulted from labeled B(a)P breakdown or from the impurities in the initial ^{14}C-B(a)P stock; therefore, this cannot be considered significant degradation of B(a)P.

B(a)P degradation was not induced by the release of nutrients from drying/wetting, the addition of carbon as straw, or the inoculation of the soil. Previous studies have concluded that low levels of B(a)P degradation could have occurred due to the limited solubility of this compound, the lack of the required enzymes found in soil microbial communities, or the lack of importance of metabolic breakdown as a major fate pathway of B(a)P in the environment (Carmichael and Pfaender, 1997). In this experiment B(a)P may have had limited bioavailability. This compound is hydrophobic therefore may have sorbed to the soil. The wetting and drying treatment, as well as the carbon amendment, were unable to stimulate the degradation of B(a)P. Perhaps this was partially due to aging of the added contaminate. The wetting and drying treatment, as well as the straw addition were not applied to the soil samples until more than a year after the diesel stock solution was added. By this time the microbial community may have had limited access to the B(a)P.

A threshold concentration of B(a)P may be required to stimulate the degradation pathway responsible for the initial oxidation of B(a)P. Perhaps the bioavailable B(a)P concentration or the diesel stock solution added to the soils was not sufficient to activate B(a)P degradation pathways. Analysis of the diesel fuel #2

detected less than 0.5 mg/L of B(a)P. Non-acclimated soils showed low rates of B(a)P if it was demonstrated at all. Kastner et al (1994) suggested that PAH degrading bacteria may be present in a soil but a minimum concentration of PAHs may be required to stimulate growth of the PAH degrading microorganisms.

B(a)P may have been incorporated into the soil. Monitoring mineralization activity is thought to underestimate the actual level of contaminant degradation that is taking place since the carbon that is not released as CO_2 is not accounted for (Morgan *et al.*, 1993). In an experiment by Kanaly et al. (1997) 50 % of ^{14}C from labeled B(a)P was recovered as CO_2. The remainder of the radioactivity was found to be dichloromethane (DCM) extractable (25 %), irreversibly bound (22.5 %), and reversibly bound (2.5%). Only 5 % or the added ^{14}C was still associated with B(a)P and this was found in the DCM extract. This experiment has not accounted for any transformation of B(a)P that may have been incorporated into the soil or the microbial biomass. Research on these soils is ongoing; therefore, destructive analysis of the soil was not possible at this time.

The ^{14}C-labels are located at the 7 and 10 positions in the structure of B(a)P. The most energetically favorable position of initial enzyme attack is at the 7-8 or the 9-10 position (Kanaly *et al.*, 1997). Though the ^{14}C labels are thought to be located where ring fission is most likely to occur, Kanaly et al. (1997) found that the majority of the added ^{14}C-B(a)P was transformed but approximately 25% of the radioactivity was irreversibly bound to the soil before it was fully metabolized.

3.7 Summary and Conclusions

B(a)P is an extremely recalcitrant compound. The indigenous microbial communities of the sixteen soils examined here were not able to degrade B(a)P over the entire duration of the incubation. The addition of an inoculum, straw as a carbon source, and wetting/drying were not able to activate the PAH metabolic pathways required for B(a)P breakdown. Perhaps the bioavailability of the contaminant was limited by sorption to the soil or the microbial community does not have the ability to degrade PAHs. The catena soils used in this experiment had not been previously exposed to hydrocarbon contamination and therefore may not have the required pathways for PAH degradation.

CHAPTER 4

Degradation of Polycyclic Aromatic Hydrocarbons in Previously Contaminated Soils

4.1 Abstract

The objective of this study was to determine if hydrocarbon contaminated sites have an adapted microbial community with an increased ability to degrade two polycyclic aromatic hydrocarbons (PAHs): anthracene and benzo(a)pyrene [B(a)P]. Soils from two previously contaminated sites were selected for a laboratory based degradation study. Microcosms were used to contain the soil samples to which ^{14}C-labeled PAHs in a diesel fuel stock solution were added. Degradation, total respiration, and volatilization were monitored during the experiment. The functional diversity of the microbial communities of these soils also was assessed.

Anthracene was degraded in all seven soils examined, though four of the soils experienced a lag time before the evolution of $^{14}CO_2$ reached significant levels. B(a)P was not degraded by any of the soils during the first three weeks of incubation. After this time the three soils that initially were able to degrade anthracene, were able to produce significant amounts of $^{14}CO_2$ from the B(a)P. The total percent of the added ^{14}C-labeled anthracene and B(a)P that was recovered as $^{14}CO_2$ was 30.5 to 48.5 and 1.4 to 53.3

respectively. At each of the two sites the soils with the highest level of hydrocarbon contamination had an increased ability to degrade PAHs. The soils with the highest respiration rates resulted in the most rapid breakdown of the PAH compounds.

4.2 Introduction

Polycyclic aromatic hydrocarbons are widespread environmental pollutants. Their carcinogenic and persistent nature has lead to various PAHs being listed as priority pollutants. Incomplete combustion of organic-carbon based materials during industrial processing, such as petroleum refining, coal gasification, and coal liquification, are responsible for the generation of PAHs (Miller *et al.*, 1988). These compounds form a family of benzene derivatives, composed of varying numbers of fused aromatic rings that range in complexity from simple two ringed structures, like naphthalene, to condensed multi-ringed substances, such as B(a)P. In the environment PAHs create potential health problems and remain persistent throughout natural ecosystems (Pothuluri and Cerniglia, 1994).

The structural stability of these compounds and the tendency to sorb to soils and sediment have resulted in great difficulties remediating PAH contaminated sites. Unsubstituted PAHs are extremely resistant to catabolism by microorganisms, and are only known to degrade in the presence of an adequate oxygen supply (Manilal and Alexander, 1991; Pothuluri and Cerniglia, 1994). Soils that have been exposed to hydrocarbon contaminants have been found to degrade PAH compounds at a higher rate than in unacclimatized soils (Wilson and Jones, 1993). B(a)P was found to be degraded

at a faster rate by bacteria isolated from soil containing B(a)P than bacteria that were isolated from uncontaminated soil (Sims and Overcash, 1983). Time and suitable conditions may be required for a microbial community to adapt to the presence of hydrocarbon contamination. The suitability of the soil conditions for the PAH degrading microorganisms will influence the persistence of these pollutants in the environment.

4.3 Objective of Study

The objective of this study was to determine if hydrocarbon contaminated sites have an adapted microbial community with an increased ability to degrade PAHs. The kinetics of anthracene and benzo(a)pyrene degradation from the two contaminated sites was to be determined. The rate of degradation was examined to determine if size, activity, or diversity of the biomass would act as biological indicators of a soil's potential to degrade PAHs.

4.4 Materials and Methods

4.4.1 Soils

The two hydrocarbon contaminated soils selected for this study were obtained from an agriculture and an industrial site[1]. A pipeline leak in October 1994 was responsible for the release of synthetic crude oil into agricultural land. Wood treatment activities at the industrial site resulted in creosote and pentachlorophenol contamination of the soil in the surrounding area. The industrial site has not been operational for a number of years.

Former knowledge of the agriculture site was used to establish a highly contaminated and a moderately contaminated sampling location, as well as a control soil from uncontaminated adjacent land. The top 15 cm of soil was sampled. The three sampling locations at the agriculture site were taken in an area cropped to wheat which was the subject of a concurrent study. The samples were similar in texture and structure (Table 4.1).

The industrial site was sampled in three locations to obtain a low, a moderate, and a highly contaminated soil sample. The soils were sampled to a depth of 15 cm. The highly contaminated soil from the industrial site was divided into two samples, resulting in four soil samples taken from this location. The first highly contaminated sample consisted of a top layer of highly organic root mat from an undetermined grass species and the second sample was taken from the top 15 cm of clay soil that was

[1] Conditions of agreement for use of the agriculture and industrial sites was based on them remaining anonymous.

underlying this area. The moderately contaminated soil was a structureless coarse textured material that may have resulted from construction fill. The soil sample with a low level of contamination was a clay that was similar to the soil found in the lower section of the highly contaminated soil. There was a lot of variability in the texture of soils found between samples (Table 4.1). Vegetation was covering all the industrial soils with the exception of the moderately contaminated soil.

Three bags of each soil type were collected and were later composited using a cement mixer. Soil samples were double bagged and kept in plastic pails at 4°C prior to the beginning of the experiment. A separate plastic pail was used for each of the soils to avoid cross contamination of the samples.

Table 4.1 Soil properties of the agriculture and the industrial contaminated soils.

Soil	pH	EC (dS/m)	Soil Moisture (%)	Field Capacity (%)	Organic Carbon (%)	Total Nitrogen (%)	Sand (%)	Silt (%)	Clay (%)	Textural Class	Sand Fraction				
											VCS	CS	MS	FS	VFS
Agriculture Control	7.6	0.86	26.4	31.2	3.71	0.32	34.7	21.2	44.1	C	5.1	11.5	16.8	25.5	41.1
Agriculture Low	7.7	3.87	32.3	36.5	4.32	0.37	22.7	69.2	8.0	SiL					
Agriculture High	6.8	0.73	26.7	33.9	4.59	0.33	26.1	36.8	37.1	CL					
Industrial Low	7.8	0.39	28.6	36.5	5.08	0.21	26.1	36.9	37.0	CL					
Industrial Moderate	7.6	0.39	26.3	22.6	20.93	0.26	74.2	14.2	11.6	SL	14.7	23.3	21.5	24.7	15.8
Industrial High Top	7.2	0.41	38.2	53.5	18.09	0.53	50.9	9.6	39.5	SCL	19.5	37.6	20.6	11.9	10.3
Industrial High Bottom	7.4	0.46	38.9	40.9	5.08	0.38	8.9	43.9	47.2	SiC					

4.4.2 Soil Properties

Soil characterization included determination of field capacity, pH, electrical conductivity, organic carbon, total nitrogen, and particle size distribution (Table 4.1).

4.4.2.1 Field Capacity. Field capacity for each of the seven soils was determined by measuring the surface soil water content of soil columns brought to field capacity. 15 cm long pieces of plastic tubing with a diameter of approximately 5 cm had one end covered with cloth and held in place firmly with an elastic band. Air dried soil that had been passed through a 2 mm sieve was added to within 2 cm of the open end of the tube. Water was added slowly and evenly over the surface of the soil until the water had moved one third of the way through the soil. The top of the apparatus was covered with Parafilm and allowed to sit for 48 hour at room temperature.

After this time the moist soil was removed from the tube and placed into a previously weighed beaker. Care was taken not to include the top layer of the soil, which could be influenced by the air-soil interface, or the soil that remained dry beneath the wetting front. The moist soil was weighed before being placed in an drying oven at 110°C for 48 hours. A desiccator was used to cool the oven dried soil before the weight was determined. The gravimetric moisture content of the soil was calculated and is referred to as the field capacity of the soil. For each soil this procedure was performed in triplicate and averaged to obtain the final field capacity values.

4.4.2.2 Soil pH . Soil pH was determined by the $CaCl_2$ method outlined by Hendershot et al. (1993). Samples of a 1:2 ratio of air dried soil to 0.01 M $CaCl_2$ were prepared in 50 mL beakers. The solutions were mixed periodically for 30 minutes and were allowed to stand for 1 hour. The pH of the supernatant was measured by use of an Accumet® model 15 pH meter and was recorded once the pH had become relatively constant. The pH for each soil was determined in triplicate and averaged.

4.4.2.3 Electrical Conductivity. Electrical conductivity of the soils was determined. Ten grams of air dried and sieved (< 2 mm) soil was weighed into 50 mL centrifuge tubes. Twenty milliliters of distilled water were added to each tube so there was a 1:2 ratio of soil to water. All tubes were sealed well and placed on a lateral shaker for 30 minutes. Immediately after the samples were removed from the shaker an Orion model 160 conductivity meter was used to determine the electrical conductivity of the soil solutions. Samples were run in triplicate and averaged (McKeague, 1978).

4.4.2.4 Organic Carbon and Total Nitrogen. Organic carbon and total nitrogen of the soils were determined by a LECO CHN-600 combustion method (LECO Corporation, St. Joseph, MI). Carbonates had to be removed from the soil samples prior to the procedure. 1 g soil samples were weighed into 30 mL beakers and 3 mL of 6 M HCl were added to each beaker. The beakers were placed on a hot plate and covered with a watch glass. Once each solution had boiled for 5 minutes it was removed from the hot

plate and let cool. The chlorides from the acid had to be removed from the soil samples to avoid interference with the analysis of the LECO CHN-600. The soil samples were transferred to 50 mL centrifuge tubes and 25 mL of water were added to each tube. They were shaken by hand for two minutes and were placed in a centrifuge at 1500 rpm for 20 minutes. The supernatant was poured into a beaker and a few drops of $AgNO_3$ were added to the solution. If a AgCl precipitate occurred then chloride remained in the soil and the washing step was repeated. The washing step was repeated until the precipitate did not form.

The soil was transferred to a 50 mL beaker and placed in an oven at 105°C for a minimum of 24 hours. The samples were cooled in a desicator and finely ground with a mortar and pestle. A four decimal place scale was used to weigh approximately 0.1200 g of sample into a small metal container. The exact weight of the soil was recorded. All samples were run through the LECO CHN-600. The samples were fully combusted and the total nitrogen and organic carbon values were recorded.

4.4.2.5 Particle Size Distribution. Particle size distribution of the soils was determined by the pipette method as outlined by Sheldrick et al. (1993). The procedure was run in duplicate for each soil. The amount of sand, silt, and clay in the duplicate samples were averaged before the textural class was determined. If the sand fraction accounted for greater than 30 % of the soil the sand fraction was passed through a set of sieves and separated into 5 size fractions.

4.4.3 Soil PAH Analysis

PAH analysis was performed on the soils by Norwest labs (Table 4.2). Though PAHs were not detected in any of the three agriculture soils sampled in 1997 for this experiment, previous PAH analysis the year following the crude oil spill indicated that PAHs were present in the areas where the low contaminated and the high contaminated soils were sampled. Naphthalene, phenanthrene, and pyrene were all detected at the area where the low level of contaminated soil was located with total PAH content varying from 0.011-0.261 mg/kg, and indeno(1,2, 3-c,d)pyrene, naphthalene, phenanthrene, and pyrene were all detected at the area where the highly contaminated soil was located with total PAH content varying from 0.538-1.177 mg/kg. Total hydrocarbon analysis (<C60) performed on these soils in 1995 also indicated that these areas were contaminated with hydrocarbons (Appendix III b and III c).

Table 4.2 PAH analysis of agriculture and industrial soils sampled in 1997 and diesel fuel used to prepare stock solutions. Units for the soil PAH analysis are mg/kg and for the diesel analysis is mg/L.

Sites	Anthracene	Benzo(a)pyrene	Total PAHs
Agriculture Control	<0.1	<0.1	nd
Agriculture Low	<0.1	<0.1	nd
Agriculture High	<0.1	<0.1	nd
Industrial Low	0.2	0.4	13.7
Industrial Moderate	1.6	4.4	94.7
Industrial High Top	4.1	7.2	168.3
Industrial High Bottom	0.6	1	28.6
Diesel	9.6	<0.5	567.7

Note: nd indicates that PAHs were not detected in that soil.

4.4.4 Soil Preparation for Degradation Study

For each of the 7 samples collected, 4 replications of the equivalent of 10 g of soil (oven dry basis) were weighed into 50 mL beakers. All samples were wet to field capacity and incubated at 20°C for approximately one month. Twice during the incubation period the soils were weighed and rewet to field capacity. All samples remained at an incubation temperature of 20°C for the duration of the experiment.

Approximately every two months the beakers of soils were removed from the experimental apparatus, weighed, and rewet to field capacity if necessary. This was to keep the soils at a constant moisture content for the duration of the experiment.

4.4.5 Microcosm Apparatus

The microcosm apparatus used to contain the 10 g soil samples consisted of a 500 mL glass jar with an air-tight fitting lid. In addition to the beaker of soil, the microcosms contained 15 mL of NaOH in a 20 mL scintillation vial to trap the CO_2, 3 mL of acidified water (pH 3) in a carrier vial to help maintain humidity, and a polyurethane foam plug suspended on a glass capillary tube to trap volatile compounds (Figure 4.1). Initially a 0.2 M NaOH solution was used to trap the CO_2 that evolved and a 0.1 M NaOH solution was used once the level of respiration slowed down.

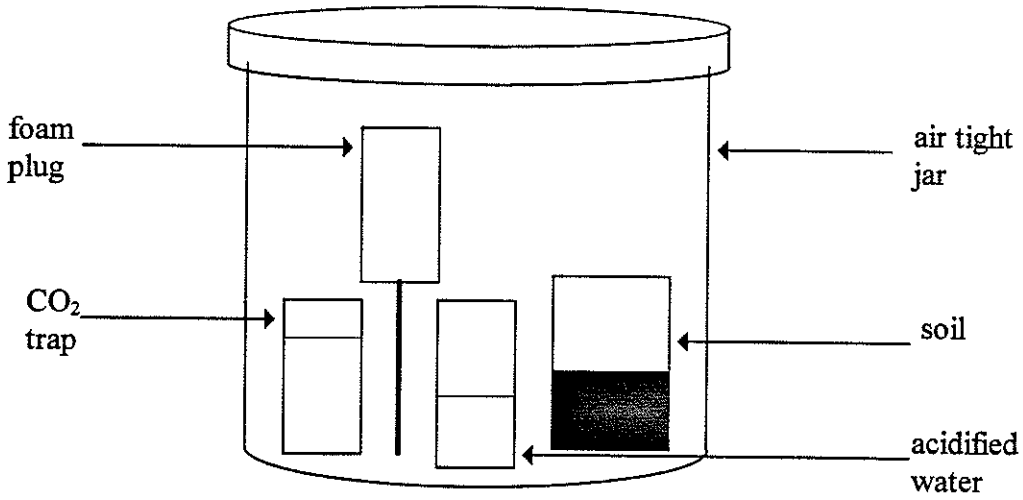


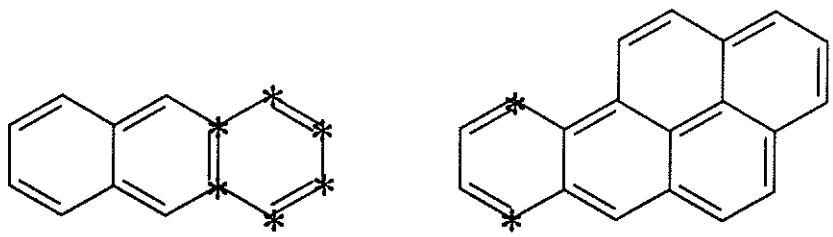
Figure 4.1 Microcosm apparatus used to contain soil samples for the degradation study.

4.4.6 ^{14}C-Labeled PAHs

Two ^{14}C-labeled PAHs were purchased: ^{14}C-1,2,3,4,4A,9A-anthracene (57 μCi/mmol at greater than 98% purity) from Sigma Chemical Co. (St. Louis, MO) and ^{14}C-7,10-B(a)P (12.2 μCi/mmol at greater than 98% purity) from Amersham International (Oakville, ON) (Figure 4.2). Stock solutions were prepared such that the addition of 1 mL to each beaker of soil would contain 5000 μg/g diesel fuel #2, 100 μg/g anthracene or 7.0 μg/g B(a)P, and hexane. Hexane was a carrier solvent intended to distribute the PAH compounds in the soil plug. ^{14}C-labeled and unlabeled PAHs were used to create the stock solution so that the level of activity added to each soil plug did not exceed 0.6 μCi. The level of PAH contamination added to each soil plug was ten times the acceptable level for an industrial site according to the CCME Soil

Quality Guidelines, March 1997. The level of diesel in the soil was such that Manitoba Environment would require remedial action to take place.

One milliliter of stock solution was added to each beaker of soil. The beakers remained in the fumehood for one hour after the addition of the PAH solution to allow the carrier solvent to volatilize. There is an inverse relationship between the number of rings in a PAH and the loss from soil via volatilization. This removal mechanism becomes negligible for PAHs with greater than two rings (Davis *et al.*, 1993; Sims and Overcash, 1983; Wilson and Jones, 1993). At the end of this period each beaker was weighed, wet to field capacity, and placed into a microcosm apparatus. All microcosms had a scintillation vial containing 15 mL of 0.2 M NaOH added and were incubated at 20°C. Later in the experiment, respiration rate of the soils decreased, therefore a 0.1 M concentration of NaOH was used to trap the evolving carbon dioxide.



^{14}C-1,2,3,4,4A,9A-Anthracene **^{14}C-7,10-Benzo(a)pyren**

Figure 4.2 Structural formula of anthracene and benzo(a)pyrene. The "*" represents the location of the ^{14}C-labels.

4.4.7 Monitoring Respiration and $^{14}CO_2$ Evolution

The NaOH traps in the microcosms were removed and replaced with new ones at 48 hours, 96 hours, and one week after the beginning of the incubation period.

Weekly trap changes were performed for the first 8 months of the experiment then the frequency of the trap changes was reduced to biweekly for the remainder of the experiment.

A 1 mL sample was removed from each 15 mL NaOH trap and placed in a 7 mL scintillation vial. Each vial had 5 mL of EcoLume Liquid Scintillation Fluid (ICN Biochemicals Inc. Aurora, OH) added to it and was set aside for a minimum of 24 hours before the level of radioactivity was determined by a Beckman LS 7500 liquid scintillation counter. Calculations were done to determine the percentage of added radioactivity present in the entire 15 mL trap.

For the first three months of the experiment total respiration was determined by performing a titration procedure on the remaining 14 mL of the NaOH traps. The vial containing each trap was emptied into a 100 mL titrator cup and rinsed well with distilled water. 5 mL of 0.5 M $BaCl_2$ was added to react with the carbonate formed from the trapped CO_2 resulting in the formation of an insoluble $BaCO_3$ precipitate. 0.1 M HCl was used to titrate the unreacted NaOH from the trap. The volume of acid required for the titration was used to determine the mg of carbon dioxide that had evolved from the soil over the incubation period.

For the remainder of the experiment a modified version of the persulfate oxidation method used for total organic carbon analysis (Technicon method # 455-76W/A) was used to determine dissolved CO_2 and carbonates (Technicon, 1978). Dissolved carbon dioxide and carbonates were determined by acidifying the sample and passing carbon dioxide across a gas diffusion membrane. The carbon dioxide was then

collected in a colour reagent containing 3.1×10^{-2} mmol of phenolphthalein per litre of solution and the change in colour was determined at 550 nm wavelength. The results were given in mg C/L of trap and then were converted into a mg C/trap basis.

4.4.8 Volatilization

Once during the experiment the polyurethane foam plugs were removed from the microcosms and replaced with new plugs. The removed foam plugs were placed in 100 mL French square bottles and 20 mL of methanol was added to each bottle (EPA §796.3400). The bottles were sealed with rubber stoppers and were placed in a shaker on low for two minutes. A 60 mL syringe was used to extract all the methanol from the foam plugs. A 1 mL sample of the methanol extract was removed from each bottle, placed in a 7 mL scintillation vial with 5 mL of EcoLume scintillation cocktail, and was counted by a liquid scintillation counter. The amount of radioactivity trapped in the foam plugs was determined as a percentage of the radioactivity initially added. The methanol extraction procedure was repeated when the experiment was terminated. The amount of radioactivity found in the two separate methanol extraction procedures were added together to determine the total percent of ^{14}C-labeled compounds that had volatilized during the experiment.

4.4.9 Soil Microbial Biomass Carbon

The chloroform fumigation-extraction method was used to determine the soil microbial biomass carbon (Voroney *et al.*, 1993). Total organic carbon of the extract

was determined by use of a persulfate oxidation method, Technicon method # 455-76W/A (Technicon, 1978). Calculations were carried out as outlined by Voroney et al. (1993) using a K_c factor of 0.25.

4.4.10 Metabolic Diversity of the Microbial Community

Commercially available Biolog GN plates are composed of 96-wells that contain 95 different carbon substrates and a control well. These plates allow for a rapid method of characterizing and classifying microbial communities based on their ability to utilize sole carbon sources (Garland and Mills, 1991). Nutrients, salts, peptone, and redox dye tetrazolium violet are in all wells of the microplates. Tetrazolium dye is reduced in response to respiratory activity and results in the formation of formazan. The purple colour that results from this reaction is used as the response variable (Winding, 1994). It is thought that Biolog plates can be used to assess quickly and effectively the functional diversity of a microbial community (Zak *et al.*, 1994).

Biolog GN microplates were purchased through BIOLOG, Inc. (Hayward, CA). Two types of 100 mL dilution bottles were prepared: the first set contained 90 mL of water agar solution (0.2%), which was prepared by dissolving 2 g of purified grade agar in 1 L of saline solution (0.85% NaCl), and the second set contained 99 mL of physiological saline solution (0.85%). Ten glass beads (5 mm in diameter) were added to each of the water agar bottles. All bottles were autoclaved prior to use, along with the pipette tips, and reagent reservoirs.

Ten grams of soil on an oven dry basis was weighed out in 4 replications for each of the 7 soils. The soils were transferred aseptically to the water agar dilution bottles, then the bottles were placed on a lateral shaker for a half hour. After shaking, 1 mL of the soil suspension was removed from each bottle and added to a saline dilution bottle. The saline dilution bottles were shaken by hand (10^{-3} dilution). A multichannel pipette was used to place 100 µl aliquots of the final dilution of soil solution into each of the 96 wells in the Biolog plates. Biolog plates were incubated at 25°C and were removed for readings at 24, 48, 72, and 96 hours. At each reading the appearance of the reduced tetrazolium dye for each 24 hour period was noted. These readings were used to determine the metabolic diversity for each soil type. Metabolic diversity is defined as the percentage of substrates that was utilized by the microorganisms.

4.4.11 Mathematical and Statistical Analysis

Degradation activity was monitored by measuring the ^{14}C that evolved as carbon dioxide and calculating it as a percent of the radioactivity that was initially added to the soil samples. CoStat (CoHort Software, Berkeley, CA) and SAS (SAS Institute Inc., Cary, NC) were used to perform one way ANOVAs and Duncan's Multiple Range tests at the 5% level on the final cumulative degradation values for anthracene and B(a)P for the two contaminated sites. Similar statistical analysis was done on the data from the metabolic diversity study, the methanol extraction procedure, and the wet oxidation procedure.

Curve fitting was performed by the use of the software package JMP IN (SAS Institute Inc., Cary, NC). Each of the 56 soil replications used in the degradation experiment had a linear, first order, and Sigmoidal curve fit to its data by use of the following equations.

$$A_t = K_L t + b_L \qquad \textbf{Equation 4.1}$$

$$A_t = A_F(1 - e^{-K_F t}) \qquad \textbf{Equation 4.2}$$

$$A_t = A_S/[1 + (A_S - b_S)/b_S \times e^{-K_S t}] \qquad \textbf{Equation 4.3}$$

Where: A_t = percent degradation at time t (days), K_L = the degradation rate constant for the linear curve ($days^{-1}$), b_L = the y intercept of the linear curve, A_F = percent of added ^{14}C that has evolved as $^{14}CO_2$ at time infinity for the first order curve, K_F = the degradation rate constant ($days^{-1}$) for the first order curve, A_S = percent of added ^{14}C that has evolved as $^{14}CO_2$ at time infinity for the sigmoidal curve, and K_S = the degradation rate constant ($days^{-1}$) for the sigmoidal curve, and b_S = the y intercept for the sigmoidal curve. All curve fitting procedures were performed by using cumulative percent degradation data at time t.

In order to compare the suitability of the fit of linear, first order, and sigmoidal curve to the degradation data a test was done to determine if there was a significant reduction in the residual sum of squares (RSS) with increasing complexity of the model. The F-statistic can be calculated by finding the difference between the RSS of two models and then dividing the difference by the residual mean square (RMS) of the more complex model. To determine if the increase in model complexity resulted in a better fit for the data this calculated F value can be compared to the suitable value in

the F table. The degrees of freedom at $P \leq 0.05$ level can be derived by using the value 1 and the number of data points minus the number of parameters (Robinson, 1985).

The K values that were determined from the best fitting curve were then used to calculate the half life for anthracene and benzo(a)pyrene in each of the seven contaminated soils. The Ks values calculated for the sigmoidal curves only referred to the active portion of the degradation curve. The lag time that existed before degradation occurred is not taken into consideration by this equation. The half life is calculated based on the percent of the chemical that was degraded. The assumption is made that this is the available portion of the compound and it has all been degraded.

$$t_{1/2} = \ln 2/K \qquad \textbf{Equation 4.4}$$

The soil sorption partition coefficients (K_d) were determined for anthracene and B(a)P with each of the seven soils using the following equations.

$$K_{oc} = 0.411\ K_{ow} \qquad \textbf{Equation 4.5}$$

(Karickhoff, 1981)

$$K_d = K_{oc} \times f_{oc} \qquad \textbf{Equation 4.6}$$

Where: K_{ow} = the octanol-water partition coefficient, K_{oc} = partitioning of the compound into the organic carbon, f_{oc} = fraction of organic carbon in the soil, K_d = soil sorption partition coefficient.

The organic carbon values that had been determined previously are in Table 4.1 and the K_{ow} values for the PAH compounds are in Table 1.2. The K_{oc} value is a property of the organic chemical and the f_{oc} value is a property of the soil. K_d values allow for an estimation of the amount of an organic chemical that will be absorbed by a particular soil and will not be available to the microorganisms.

4.4.12 Destructive Analysis of Soil Samples

Once the rate of PAH degradation leveled off, the location of the remainder of the radioactivity was to be determined. From each microcosm 1 g of soil sample, on an oven dry basis, was used for an additional Biolog plate study. The purpose of this study was to determine if the additional contamination and the conditions of the experiment had altered the ability of the soil microbial community to function. The remaining 9 g of soil were used for a sequential extraction procedure. First a microbial biomass extraction was performed followed by a methanol extraction and a wet oxidation procedure. Following a degradation experiment Maurice (1998) performed a series of extractions to locate ^{14}C-labeled phenanthrene: water, methanol, dichloromethane, and wet oxidation. The methanol extraction and wet oxidation procedures recovered the highest percentage of the added radioactivity

Microbial biomass extraction would allow for the determination of ^{14}C that may have been incorporated into the biomass. The methanol extraction would contain ^{14}C-labeled PAHs and degradation intermediates that were weakly associated with the soil and are thought to have limited bioavailable. The wet oxidation procedure results in total digestion of soil organic carbon so that any ^{14}C that may be associated with the soil is released and can be accounted for. This fraction of radioactivity is considered to be unavailable to the microbial community and may have undergone irreversible sorption or humification (Maurice, 1998).

4.4.12.1 Biolog Plate Procedure. Biolog GN microplates were purchased through BIOLOG, Inc. From each beaker a 1 g sample of soil was removed (on an oven dry basis) and was transferred by aseptic technique to a dilution bottle containing 99 mL of agar-saline solution and ten glass beads. All dilution bottles were shaken on a lateral shaker for a half hour. After shaking a 10 mL sample was removed and added to a second set of dilution bottles that contained 90 mL of saline solution. The saline dilution bottles were shaken by hand (10^{-3} dilution). Both sets of dilution bottles were prepared with the same concentrations of solutions as mentioned previously. All bottles were autoclaved prior to use, along with the pipette tips, and reagent reservoirs. Inoculation of the Biolog plates and plate readings were performed as previously mentioned. At the end of the 96 hour incubation period the metabolic diversity was calculated for each of the soils.

4.4.12.2 Biomass Extraction Procedure. The remainder of the soil (9 g on an oven dry basis) was split into two samples that were placed in Teflon centrifuge tubes. Teflon tubes were used to avoid adsorption of the ^{14}C-labeled compound to the container. The chloroform fumigation-extraction method was used to determine the amount of ^{14}C that was associated with the soil microbial biomass (Voroney *et al.*, 1993). One set of the soil samples was subjected to chloroform fumigation for 24 hours. The same extraction procedure was performed on both the fumigated and the non-fumigated soil samples. Each tube had 9 mL of 0.5 M K_2SO_4 (1:2 ratio of soil to K_2SO_4) added to it and was shaken on a lateral shaker for one hour. Instead of filtering the extract as outlined by

Voroney et al. (1993) all samples were centrifuged at 1200 rpm for 20 minutes. 1 mL sample of the extract was removed, placed in a 7 mL scintillation vial with 5 mL of Ecolume, and counted by the liquid scintillation counter. The radioactivity associated with the extract from the non-fumigated biomass is considered the salt extractable fraction of the radioactivity and the radioactivity associated with the biomass was determined by calculating the difference in radioactivity associated with the fumigated and the non-fumigated samples.

4.4.12.3 Methanol Extraction Procedure. Following the microbial biomass procedure the remaining extract was removed from the centrifuge tubes that contained the fumigated soil samples and a methanol extraction was performed on these soils. 22.5 mL of methanol was added to each centrifuge tube; they were then vortex for 10 seconds and placed on a lateral shaker for 24 hours. The samples were then centrifuge at 1200 rpm for 20 minutes. A 1 mL sample of the methanol extract was removed for determination of the level of radioactivity associated with this fraction. Calculations were done to determine the percent of the added radioactivity that would be in the methanol extract if this procedure had been performed on the entire 10 g soil sample. The remainder of the methanol extract was poured out of the centrifuge tubes and the excess methanol was removed by drying the soil at 60°C for a minimum of 24 hours in preparation for the wet oxidation procedure.

4.4.12.4 Wet Oxidation Procedure. A wet oxidation procedure was used to convert the total soil carbon to carbon dioxide (Voroney *et al.*, 1991). This allowed for any ^{14}C-

labeled carbon that had become associated with the soil to be released and accounted for. Depending on the percentage of carbon of each soil either 0.4 or 0.8 g of finely ground soil from each centrifuge tube was weighed into thick walled digestion tubes. A digestion mixture of CrO_3, concentrated H_3PO_4, and H_2SO_4 was prepared as outlined by Voroney et al. (1991). Six milliliters of the digestion solution was added to each tube. Immediately following this a glass rod was placed in each tube in order to support a 7 mL scintillation vial with either 3 mL or 2 mL of 2M NaOH depending on the carbon level of the soil. A #49 Rubber Suba Seal stopper was used to seal each tube tightly. In a fumehood the tubes were placed in the digestion block and were heated to 130°C for 1 hour. Once the digestion was complete the apparatus was left to cool overnight to allow for complete CO_2 adsorption. The CO_2 traps were removed from the digestion tubes and a 0.3 mL portion of the CO_2 trap was place in a 7 mL scintillation vial with 5 mL of Ecolume for determination of the radioactivity. The full volume of the base trap would be too concentrated for the scintillation cocktail. Calculations were done to determine the percent of added radioactivity that was contained in the entire NaOH trap and the full amount of soil in the microcosm.

4.4.13 Soil Respiration and Metabolic Diversity

During preparation for the degradation study an additional set of microcosms was constructed with the contaminated soils. These soils were treated in a similar manner as those prepared for the degradation study but diesel stock solution was not added to this set of soils. The microcosms were kept at 20°C and soil respiration was monitored for the same duration as the degradation experiment. NaOH traps, of the same volume and

concentration as those used in the degradation study, were used to trap the CO_2 that evolved. These traps were periodically changed and analyzed for carbon dioxide content using the same methods as previously mentioned for the degradation experiment.

At the end of the degradation experiment this set of soils was used to perform an additional Biolog plate experiment using the same procedure that was performed on a sample of these soils prior to the beginning of the degradation study. The purpose of this set of microcosms was to monitor the level of respiration from these soils that did not have diesel to act as an additional carbon source. The Biolog plate study was to determine if the incubation conditions altered the metabolic diversity of the soil microbial communities.

4.5 Results

4.5.1 Degradation Study

Three of the seven contaminated soils were able to degrade anthracene without a lag period. These soils were the two agriculture contaminated samples and the top layer of the highly contaminated industrial soil (Figure 4.3). After approximately three weeks the rate of metabolism started to slow down in these three soils. At this time $^{14}CO_2$ production started to reach significant levels in the other four soils from these two sites. Total anthracene degradation leveled off between 40 and 50 % for all soils with the exception of the moderately contaminated industrial sample. Total degradation in this soil reached only 30.5 % of the added radioactivity. The amount of anthracene degradation was not found to be significantly different between the agriculture soils,

and only the moderately contaminated sample was significantly lower from the other industrial soils (Table 4.3). The structureless coarse texture of this soil and its poor structure may not have provided a suitable microbial environment. The field capacity of the moderately contaminated industrial soil was 22.6 % while the field capacity of the other soils ranged from 31.2 to 53.5 %.

Initially the seven soils were not able to degrade B(a)P. Three weeks into the experiment the two agriculture contaminated soils and the top layer of the highly contaminated industrial soil started to produce significant levels of $^{14}CO_2$ (Figure 4.4). The bottom layer of the highly contaminated site, as well as the industrial soil with the low level of contamination, experienced a lag time of approximately 140 days before B(a)P degradation was evident. The moderately contaminated industrial soil and the agriculture control soil did not degrade a significant amount of B(a)P by the end of the incubation period. The significant differences in B(a)P degradation found at the two sites were related to the level of hydrocarbon contamination. In the agriculture site the amount of B(a)P degradation increased as the level of contamination increased. A similar trend was seen at the industrial site. The top layer of the highly contaminated soil had the highest level of B(a)P degradation (Table 4.4).

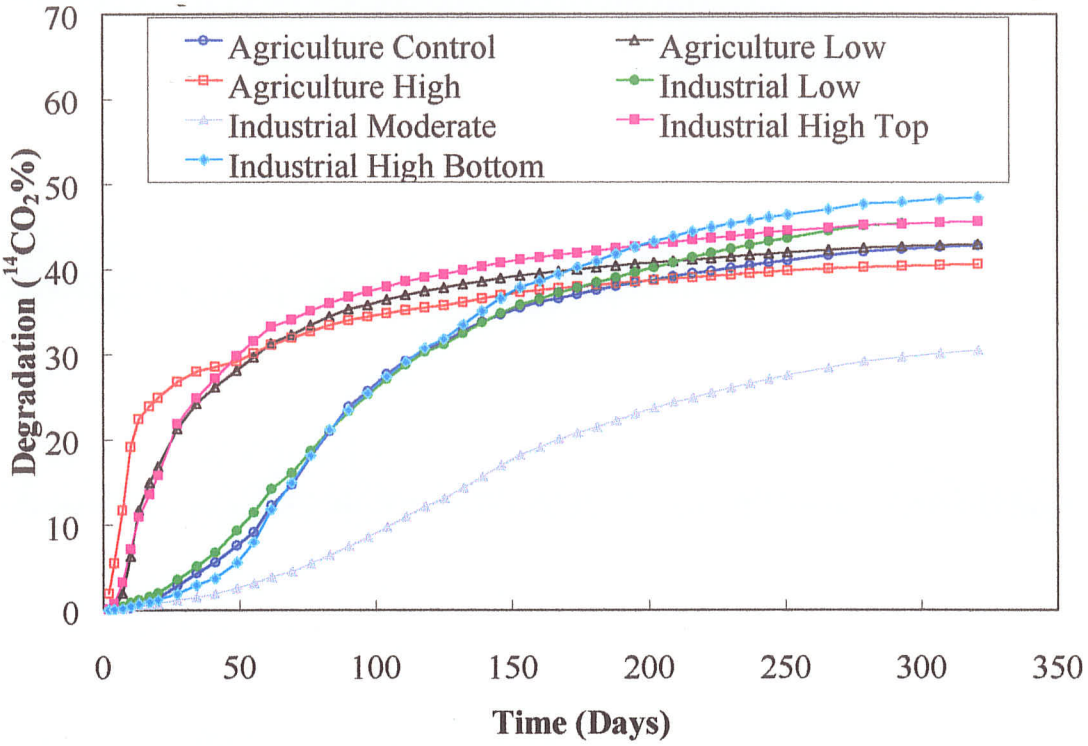


Figure 4.3 Cumulative degradation of anthracene in the contaminated soils. Points represent the mean of four replicates.

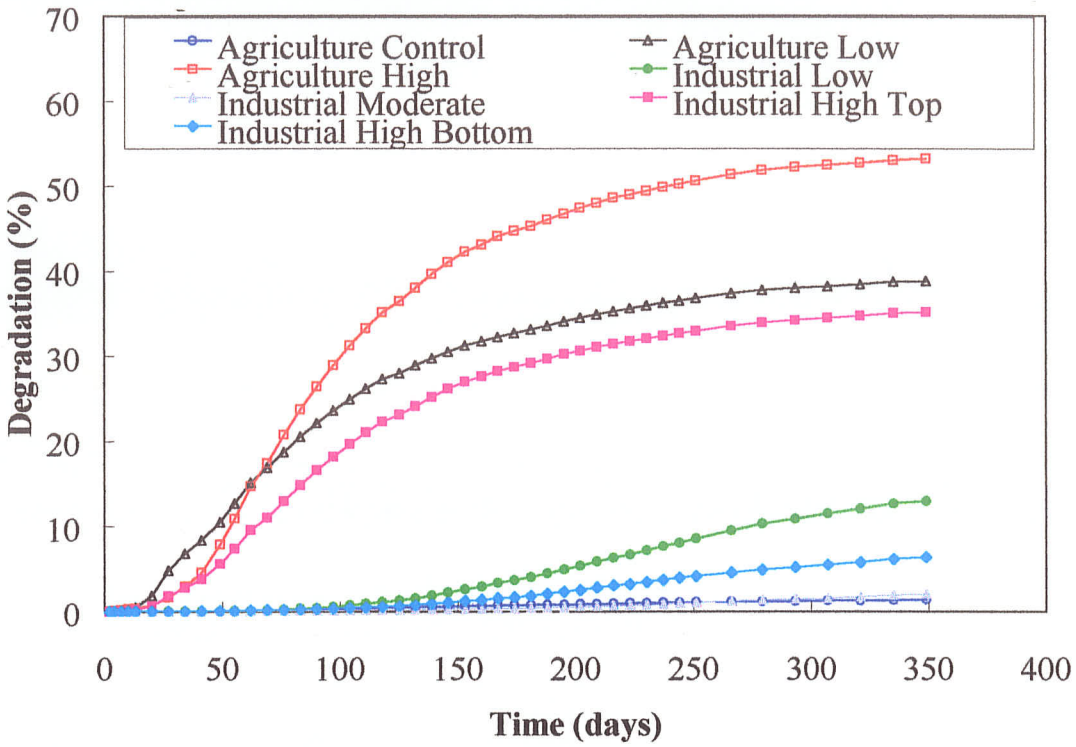


Figure 4.4 Cumulative degradation of benzo(a)pyrene in the contaminated soils. Points represent the mean of four replicates.

Table 4.3 Percent of radioactivity added as ^{14}C-labeled anthracene recovered in each fraction ($\alpha = 0.05$).

Site	Degradation	Volatilization	Salt Extractable	Microbial Biomass	Methanol Extractable	Wet Oxidation	Total
Agriculture Control	42.9	1.42a	0.47b	0.32	5.3a	5.15a	55.6 ± 0.9
Agriculture Low	43.0	0.74b	0.40b	0.26	1.8c	4.73a	50.9 ± 5.0
Agriculture High	40.7	0.20c	0.89a	0.10	3.2b	2.81b	47.9 ± 4.0
	ns	**	***		***	***	
Industrial Low	46.1a	1.57a	0.25a	0.24	2.8bc	3.92b	54.9 ± 2.6
Industrial Moderate	30.5b	1.65a	0.08c	0.19	11.2a	6.26a	49.9 ± 3.6
Industrial High Top	45.7a	0.66b	0.07c	0.24	2.2c	4.02b	52.9 ± 2.0
Industrial High Bottom	48.5a	2.06a	0.17b	0.36	3.7b	1.81c	56.6 ± 1.5
	***	**	***		***	**	

Table 4.4 Percent of radioactivity added as ^{14}C-labeled benzo(a)pyrene recovered in each fraction ($\alpha = 0.05$).

Site	Degradation	Volatilization	Salt Extractable	Microbial Biomass	Methanol Extractable	Wet Oxidation	Total
Agriculture Control	1.4c	0	0.09a	0.02	11.2a	30.94a	43.7 ± 14.1
Agriculture Low	38.9b	0	0.07ab	0.00	5.0b	17.48ab	61.5 ± 6.3
Agriculture High	53.3a	0	0.05b	0.04	6.0b	10.63b	70.0 ± 6.7
	***		*		***	*	
Industrial Low	12.1b	0	0.07	0.02	14.9a	29.41b	56.5 ± 17.1
Industrial Moderate	2.0d	0	0.06	0.04	8.9b	56.78a	67.8 ± 17.7
Industrial High Top	35.2a	0	0.02	0.03	11.8ab	19.28b	66.3 ± 8.7
Industrial High Bottom	6.4c	0	0.06	0.00	10.2b	30.91b	47.6 ± 13.7
	***		ns		*	**	

All five soils that were able to degrade both PAH compounds degraded the three ringed anthracene prior to producing significant levels of $^{14}CO_2$ from the five ringed B(a)P. The highly contaminated agriculture soil was able initially to degrade the anthracene. The rate of anthracene degradation started to slow down three weeks into the experiment but it was at this time that the more complex B(a)P started to degrade (Figure 4.5). All three soils that were able to initially degrade the anthracene followed a similar trend. The industrial site with the low level of contamination experienced a lag time of three weeks before anthracene was degraded and an even longer lag time of approximately 140 days before degradation of B(a)P was evident (Figure 4.6). The bottom layer of the highly contaminated industrial site also experienced similar lag periods before degradation was evident. These results tend to suggest that the rate of PAH degradation is influenced by the complexity of the PAH compounds. The simpler PAHs are degraded before the more complex PAHs.

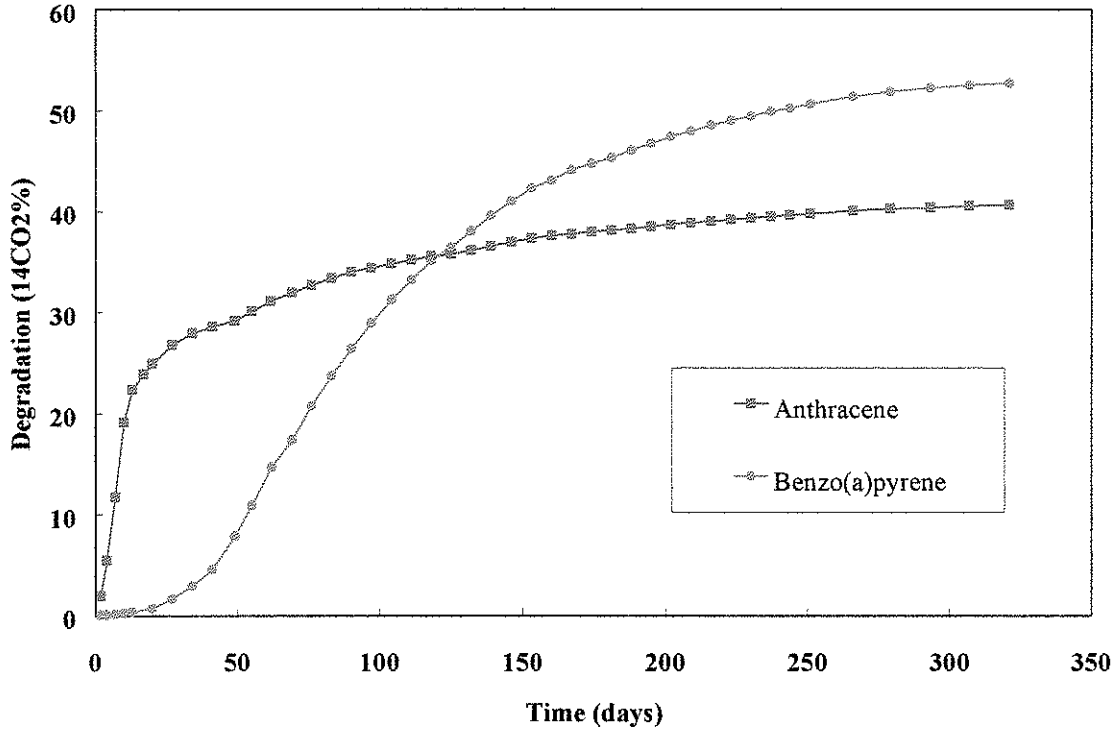


Figure 4.5 Cumulative degradation of PAHs in the highly contaminated agriculture site following the order of complexity of the compound. Points represent the mean of four replicates.

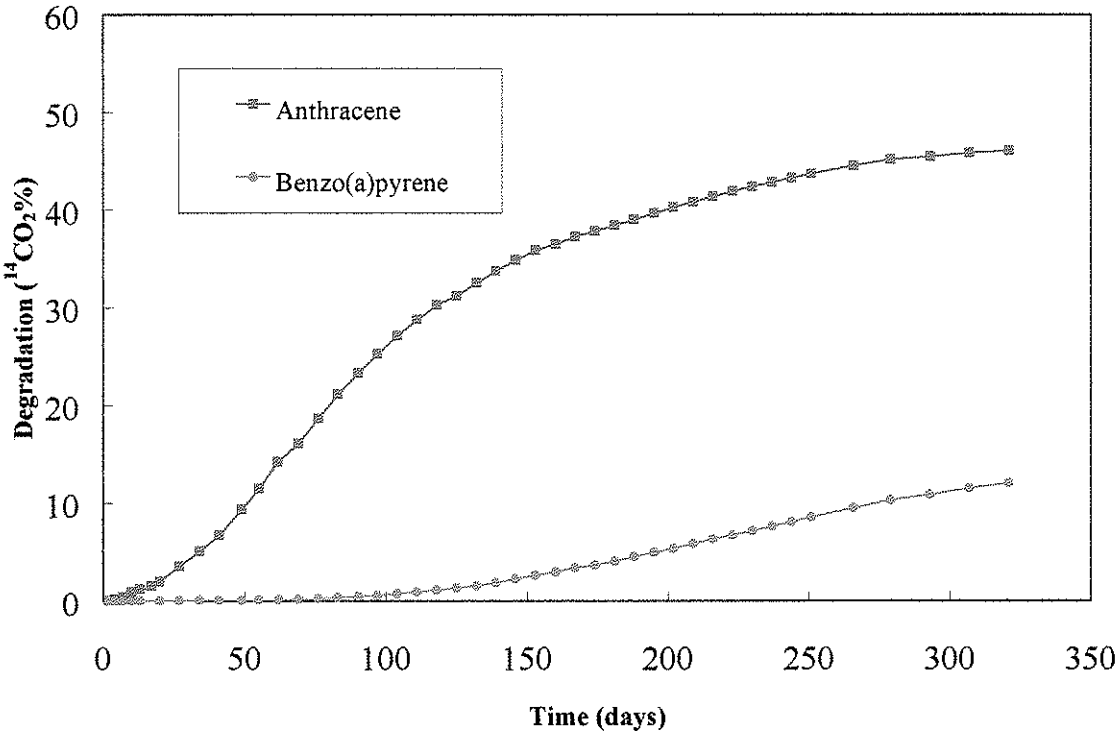


Figure 4.6 Cumulative degradation of PAHs in the low level of contaminated soil at the industrial site following the order of complexity of the compound. Points represent the mean of four replicates.

Curve fitting analysis of the anthracene degradation data indicated that two separate trends occurred (Table 4.5). The three contaminated soils that did not experience a lag time prior to the degradation of anthracene fit the first-order model while the other 4 soils were best suited to the sigmoidal model. In all seven soils the rate of anthracene degradation leveled off by the end of the incubation period. Half life values for anthracene degradation range from 17.5 days to 32.2 days.

Table 4.5 Curve fitting analysis of the anthracene degradation results and half life values. Values shown in table represent the mean of four replicates.

Sites	Best Fit Model	K_F or K_S	A_F or A_S	Mineralization Half Life (days)
Agriculture Control	Sigmoidal	0.040	39.99	17.5
Agriculture Low	First Order	0.023	41.55	31.7
Agriculture High	First Order*	0.045	38.63	18.9
		(0.023)	(37.46)	
Industrial Low	Sigmoidal[α]	0.032	43.04	21.9
		(0.0088)	(49.61)	
Industrial Moderate	Sigmoidal	0.025	29.39	28.2
Industrial High Top	First Order	0.022	43.87	32.2
Industrial High Bottom	Sigmoidal	0.042	46.11	17.9

* The RSS indicated that one of the replications was best suited to the sigmoidal model therefore the values are the average of the other three replications that fit the first-order model. Bracketed values are for the replication that fit the sigmoidal curve.
[α] The RSS indicated that one of the replications was best suited to the first-order model therefore the values are the average of the other three replications that fit the sigmoidal model. Bracketed values are for the replication that fit the first-order curve.

Analysis of the RSS values indicate that the sigmoidal model is the best fit for the B(a)P degradation data (Table 4.6). The agriculture control and the moderately contaminated industrial site have had extremely low levels of degradation ($\leq 2\%$) therefore it is not clear whether radioactivity is associated with the compound of interest or radioactive impurities. The half life values for benzo(a)pyrene ranged from 22.4 days to 40.8 days.

Table 4.6 Curve fitting analysis of the benzo(a)pyrene degradation results and half life values. Values shown in table represent the mean of four replicates.

Sites	Curve	K_L, K_F, or K_S	b_L, A_F, or A_S	Mineralization Half Life (days)
Agriculture Control	Linear[δ]	0.005	-0.087	
Agriculture Low	Sigmoidal[α]	0.033	35.33	22.6
		(0.0087)	(43.44)	
Agriculture High	Sigmoidal	0.033	50.20	20.7
Industrial Low	Sigmoidal[β]	0.023	13.64	22.4
Industrial Moderate	Sigmoidal[δ]	0.017	2.57	30.7
Industrial High Top	Sigmoidal	0.031	33.03	40.8
Industrial High Bottom	Sigmoidal*	0.023	7.57	32.7
		(0.010)	(-0.30)	

[α] The RSS indicate that two of the replications were best suited to the first order model and the other two were best suited to the sigmoidal model. The values displayed in the table are for the sigmoidal model and the bracketed values are for the first-order model.

[β] Three replications were used to calculate the values shown in the table. The fourth replication was used to create an inoculum for an additional study.

[δ] These soils have displayed ≤ 2% total degradation of the PAH compound.

[*] The RSS indicated that one of the replications was best suited to the linear model therefore values shown in the table are the average of the other three replications that fit the sigmoidal model. The bracketed values are for the replication that fit the linear model.

Anthracene and benzo(a)pyrene have high K_d values for all seven soils (Table 4.7). This indicates that both of these PAHs have a tendency to sorb to the soil. Sorption may have limited the availability of these compounds to the soil microorganisms.

Table 4.7 Calculated soil sorption partition coefficients (K_d) with anthracene and benzo(a)pyrene for the agriculture and industrial soils. The K_{ow} value for the PAH compounds (Schwarzenback et al., 1993) and the fraction of organic carbon determined from field samples were used in Equation 4.5 and 4.6.

Site	Anthracene log K_d	Benzo(a)pyrene log K_d
Agriculture Control	2.72	4.09
Agriculture Low	2.79	4.16
Agriculture High	2.82	4.19
Industrial Low	2.86	4.23
Industrial Moderate	3.47	4.84
Industrial High Top	3.41	4.78
Industrial High Bottom	2.86	4.23

4.5.2 Volatilization

Methanol extraction of the foam plugs at the end of the experiment showed that volatilization of ^{14}C-labeled compounds had taken place in the microcosms to which anthracene had been added but no volatilization was evident in the B(a)P microcosms. The results from the ^{14}C-anthracene microcosms can be seen in Table 4.3. It appears that the soils with the highest level of volatilization during the experiment were the samples that experienced a lag time prior to degrading anthracene. The agriculture highly contaminated soil and the industrial highly contaminated top layer had a significantly lower amount of volatilization than the other soils at each site. Volatilization of PAHs from soils does not appear to be a major loss pathway from the environment. In most cases the amount of volatilization was less than 2% of the added radioactivity.

4.5.3 Respiration

Respiration followed a similar trend to the degradation of the PAH compounds (Figure 4.7 and Figure 4.8). Although there is a lot of variability in the respiration data, there was a consistent trend between the level of microbial activity and PAH degradation. The three highly contaminated soils that had a higher ability to degrade PAHs had the highest level of total respiration. The moderately contaminated industrial site that had the lowest overall PAH degradation also had the lowest level of microbial activity.

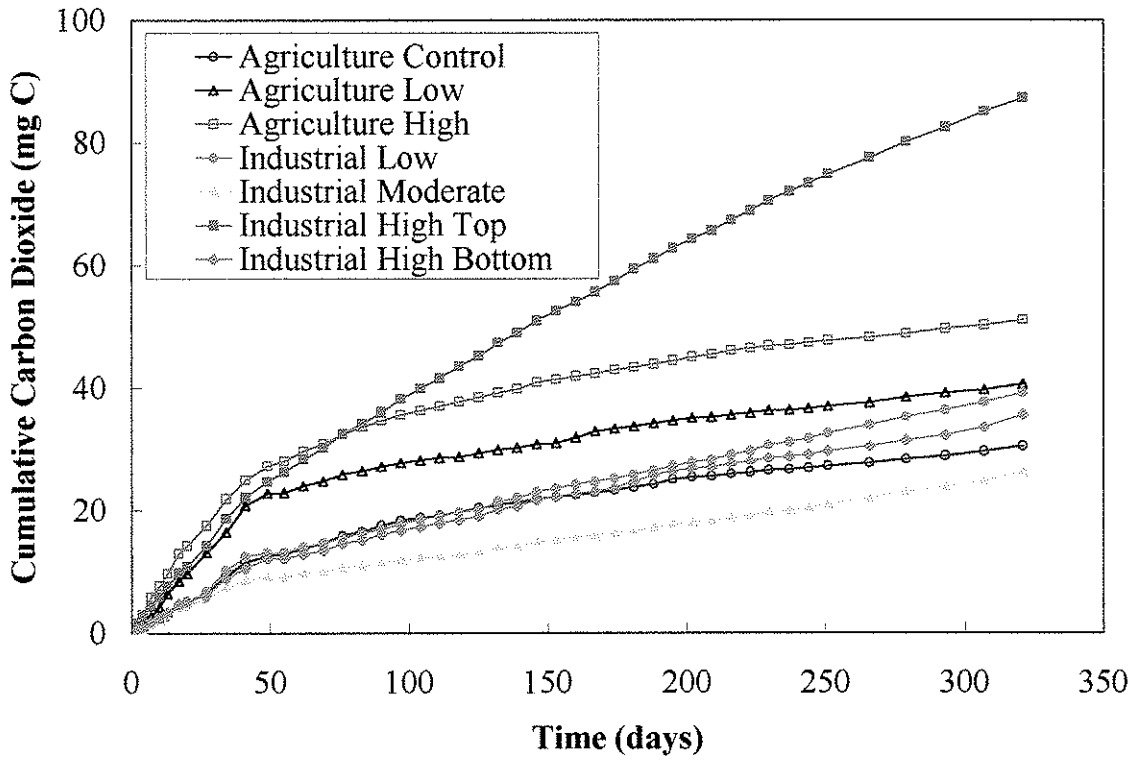


Figure 4.7 Cumulative soil respiration from the anthracene degradation study. Points represent the mean of four replicates.

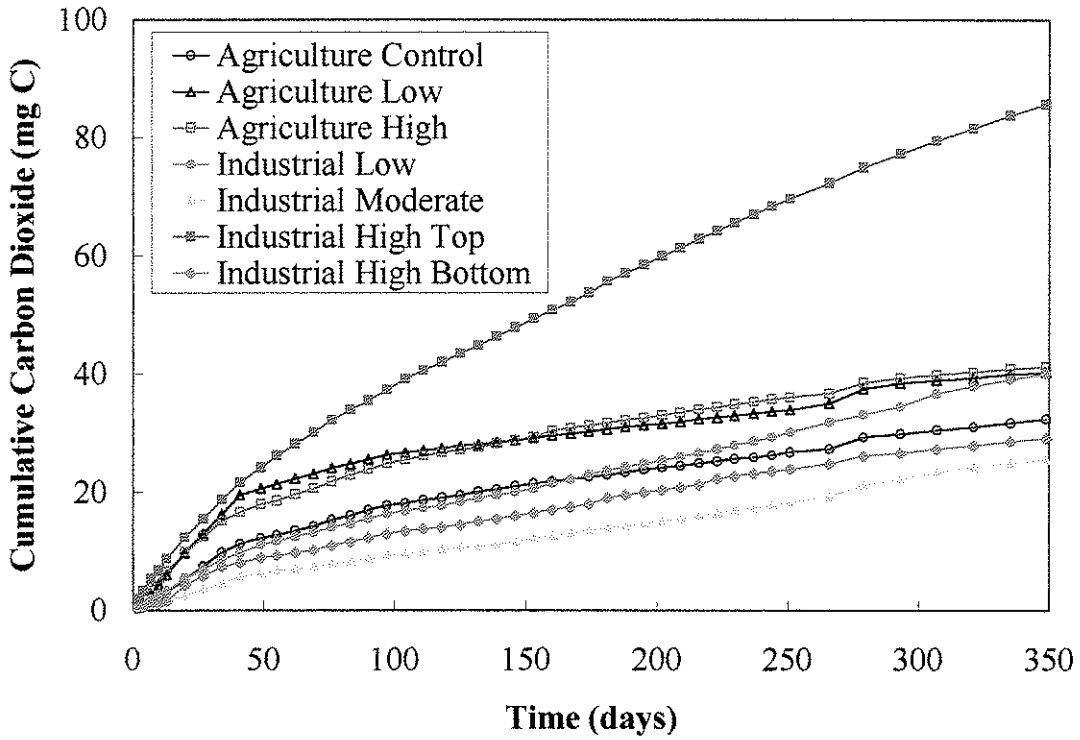


Figure 4.8 Cumulative soil respiration from the benzo(a)pyrene degradation study. Points represent the mean of four replicates.

Soil respiration results from the additional set of microcosms used to monitor activity of the microbial community of the contaminated soils can be seen in Figure 4.9. The highly contaminated top layer from the industrial site has the highest microbial activity. At the beginning of the incubation period the highly contaminated agriculture soil also had a higher level of CO_2 evolution than the majority of the soils but after approximately 60 days the rate of respiration slowed down. With the exception of the highly contaminated top layer of the industrial site few differences in microbial activity could be seen between soils by the end of the incubation period.

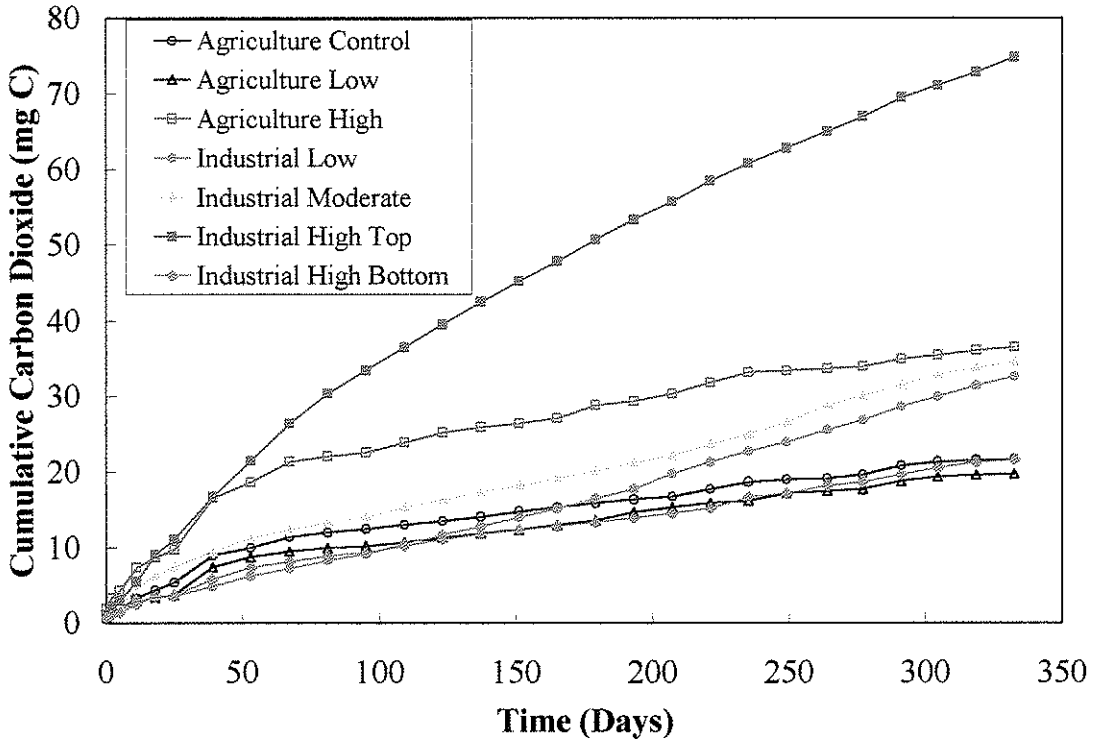


Figure 4.9 Cumulative soil respiration from the additional set of microcosms used to monitor activity of the soils without additional contamination. Points represent the mean of four replicates.

4.5.4 Microbial Biomass

The relationship between the size of the microbial biomass and the ability of a soil to degrade PAHs was inconsistent (Table 4.8). The agriculture site displayed a decrease in the size of the microbial biomass as the level of hydrocarbon contamination increased. Although a smaller microbial community resulted from the increase in contamination the overall microbial activity and PAH degradation was higher in these soils. The industrial site had an increase in microbial biomass with increasing contamination. In this site the soil with the highest level of contamination and the largest microbial biomass also had the highest microbial activity and PAH degradation.

Results from the moderately contaminated industrial soil indicate that this soil had a low microbial population size and microbial activity.

Table 4.8 Soil microbial biomass carbon for the agriculture and industrial sites.

Site	Carbon (μg/g soil)
Agriculture Control	1234 ± 100
Agriculture Low	967 ± 87
Agriculture High	288 ± 165
Industrial Low	409 ± 10
Industrial Moderate	100 ± 13
Industrial High Top	1498 ± 166
Industrial High Bottom	1294 ± 65

4.5.5 Metabolic Diversity

Biolog plate studies performed with these soils prior to the degradation study found there to be no differences in metabolic diversity between the three agriculture sites at the 5 % significance level (Table 4.9). The number of substrates the microbial community was able to utilize was not found to be significantly different between the three levels of contaminated soils. Significant differences would have been found if a 10 % level had been chosen (value was $P = 0.0685$).

The top layer of the highly contaminated industrial site was found to have a significantly higher metabolic diversity than the other three soils from this site (Table 4.9). Metabolic diversity was not found to be a consistent indicator of the ability of a soil to degrade PAHs. Though few differences in metabolic diversity were found

between these soils, there were definite differences in their ability to degrade anthracene and B(a)P.

Table 4.9 Metabolic diversity of agriculture and industrial soils prior to the degradation experiment. All values in table are the mean of four replications ($\alpha = 0.05$).

Site	Metabolic Diversity (%)
Agriculture Control	84 ± 3.7
Agriculture Low	84 ± 2.3
Agriculture High	78 ± 4.9
	ns
Industrial Low	68 ± 8.7b
Industrial Moderate	71 ± 3.8b
Industrial High Top	87 ± 1.3a
Industrial High Bottom	72 ± 4.6b
	**

At the end of the degradation study an additional metabolic diversity study was performed with the soils from the microcosms. The highly contaminated soil from the agriculture site had a significantly higher metabolic diversity for both the anthracene and the benzo(a)pyrene contaminated sets of soil in comparison to the other agriculture sites. In the anthracene contaminated soils the top layer of the highly contaminated industrial soil was found to have a significantly higher metabolic diversity than the rest of the soils at this site. This was also found to be true for the B(a)P contaminated soils from the industrial site except that the moderately contaminated soil was found to have a significantly lower metabolic diversity than the other 3 soils.

The metabolic diversity study performed with these soils prior to the degradation study had a standard deviation of less than 10 % between replications for all seven soils (Table 4.9). The variability in metabolic diversity values from the Biolog plate study performed on the soils after the degradation study had much larger variability between replications (Table 4.10).

Table 4.10 Metabolic diversity of agriculture and industrial soils after the degradation experiment. All values in table are the mean of four replications ($\alpha = 0.05$).

Site	Metabolic Diversity	
	Anthracene (%)	Benzo(a)pyrene (%)
Agriculture Control	18.5 ± 8.0b	16.4 ± 14.2b
Agriculture Low	25.3 ± 23.3b	34.9 ± 14.3b
Agriculture High	70.3 ± 4.4a	64.1 ± 2.5a
	**	***
Industrial Low	26.0 ± 19.7b	43.4 ± 13.9b
Industrial Moderate	7.8 ± 11.5b	15.4 ± 6.8c
Industrial High Top	58.1 ± 10.4a	72.4 ± 4.0a
Industrial High Bottom	27.3 ± 18.7b	42.2 ± 15.0b
	**	***

A Biolog plate study was performed on the soils from the additional set of microcosms to determine if the incubation conditions had altered the metabolic diversity of the soil microbial communities (Table 4.11). The highly contaminated agriculture soil had a significantly higher metabolic diversity than the other two soils from this site. The top layer of the highly contaminated soil from the industrial site was found to have a higher metabolic diversity than the other soils at this site. The moderately contaminated industrial soil had an extremely low metabolic diversity of 3.1%. The percentage of

substrates used by the microbial community from the incubated soils was considerably less than had been found in the Biolog plate study performed on the soils prior to beginning the degradation experiment.

Table 4.11 Metabolic diversity of agriculture and industrial soils from the additional set of microcosms used to monitor respiration of the soils. All values in table are the mean of four replications ($\alpha = 0.05$).

Site	Metabolic Diversity (%)
Agriculture Control	16.7 ± 3.1b
Agriculture Low	21.6 ± 4.7b
Agriculture High	64.3 ± 2.5a
	***
Industrial Low	30.2 ± 16.6b
Industrial Moderate	3.1 ± 1.2c
Industrial High Top	70.1 ± 5.9a
Industrial High Bottom	24.7 ± 10.6b
	***

4.5.6 Extraction Procedures and Wet Oxidation

Less than one percent of the ^{14}C-anthracene initially added to the soils was found associated with the salt extraction or the microbial biomass (Table 4.3). Although statistical analysis found there to be significant differences between the level of radioactivity found in each of these fractions, the amount detected was extremely small. The methanol extraction contained a greater amount of the radioactivity than the salt extraction or the microbial biomass procedure. At the agriculture site the control soil had the highest amount of radioactivity in the methanol extract. The moderately

contaminated industrial soil was the only one at this site to have greater than 10 % of the added ^{14}C detected in this extract.

The wet oxidation procedure released less than 10 % of the radioactivity added as ^{14}C-labeled anthracene from the soils. At the agriculture site the highly contaminated soil had significantly less ^{14}C released from the soil oxidation. The moderately contaminated industrial site had the most radioactivity released upon digestion and the bottom layer of the highly contaminated soil had the least.

Problems occurred with the wet oxidation procedure that may have lead to an underestimation of the amount of radioactivity associated with the soil. Small crystals formed in the NaOH used to trap the CO_2 during the digestion procedure. If the crystals contained the ^{14}C-labeled carbon and settled to the bottom of the vial, subsampling the solution would have underestimating the ^{14}C that was associated with the soil. One milliliter of distilled water was added to the NaOH traps from the highly contaminated top layer of the industrial soil and the moderately contaminated industrial soil. The traps were set on a shaker at 40°C for 24 hours, let cool to room temperature, and were subsampled for determination of the radioactivity. Approximately twice the amount of radioactivity was detected in all four replications for both of the soil samples. The wet oxidation procedure detected 11.3 % and 15.6 % of the added radioactivity in the highly contaminated top layer and the moderately contaminated industrial soils respectively. If these wet oxidation values are taken into consideration then the total recovery of ^{14}C for these two soils would be approximately 60 % of the added radioactivity. This data suggests that the amount of radioactivity

detected by the wet oxidation study for the anthracene contaminated soils has underestimated the true amount of ^{14}C-labeled associated with the soils.

An extremely small percent of the ^{14}C-benzo(a)pyrene initially added to the soils was found associated with the salt extraction or the microbial biomass (Table 4.4). Although statistical analysis found there to be significant differences between the level of radioactivity found in each of these fractions, the amount detected was extremely small and cannot be considered significant. The methanol extraction contained a greater amount of the radioactivity than the salt extraction or the microbial biomass procedure. The control soil from the agriculture site had a significantly higher amount of ^{14}C in the methanol extract than was found in this fraction from the contaminated soils at this site. At the industrial site the low level of contaminated soil had the most radioactivity in the methanol extract although this soil was not significantly different from the top layer of the highly contaminated soil.

Crystals formed in the NaOH traps during the wet oxidation procedure performed on the ^{14}C-B(a)P set of soils. All NaOH traps were treated as outline above for the highly contaminated top layer and the moderately contaminated industrial soils containing the ^{14}C-labeled anthracene. 30.9 % of the radioactivity added as ^{14}C-B(a)P was released from the agriculture control soil by the wet oxidation procedure. The agriculture control soil had a significantly higher amount of radioactivity associated with it than was associated with the highly contaminated agriculture site (10.6 %). At the industrial site the moderately contaminated soil had the most ^{14}C associated with it (56.8 %) and the top layer of the highly contaminated soil had the least (19.3 %).

4.6 Discussion

4.6.1 PAH Degradation Results

The results from the degradation study suggest that the rate of anthracene and B(a)P degradation is related to the level of previous hydrocarbon contamination. The three soils with the highest level of contamination were able initially to degrade the anthracene and experienced a shorter lag time before degrading B(a)P.

Anthracene degradation leveled off around 40-50 % for all soils with the exception of the moderately contaminated industrial soil. Richnow et al. (1995) performed a degradation study with ^{14}C-labeled anthracene. 25 % was recovered as $^{14}CO_2$, 5 % was recovered by organic solvent extraction, and 50 % was bound to the soil. The K_d values in Table 4.7 calculated for these sites indicate that sorption of PAHs to these soils is a likely fate and may have decreased the availability of these compounds to the microbial community.

The highly contaminated agriculture soil was able to degrade an extremely high level of B(a)P. Over 50 % of the added radioactivity was recovered as $^{14}CO_2$. Only three of the seven soils had greater than 30 % degradation. In a mineralization study by Grosser et al. (1991) in all soils except for one less than 5 % B(a)P degradation was experienced. They believed that the reason for the low level of B(a)P degradation was due to the partitioning of the compound into the sediment which limited bioavailability.

Grosser et al. (1991) suggested that the total organic carbon of soil and sediment may play an important role in the availability of compounds to

microorganisms. Although there were differences in the organic carbon content of the agriculture and industrial soils, the percent of anthracene degraded was approximately the same with the exception of the moderately contaminated industrial site. The top layer of the highly contaminated soil and the moderately contaminated soil had the highest level of organic carbon at the industrial site. Although the moderately contaminated soil experienced little degradation of B(a)P, the highly contaminated top layer experienced significant degradation of this compound. The agriculture control soil also experienced low levels of B(a)P degradation but had the lowest organic carbon content of all the soil samples (Table 4.1). The results of this study suggest that a factor other than total organic carbon influences the degradation of PAH compounds in soil.

The degradation data also indicated that complexity of the PAH influenced the sequence of degradation. There was a consistent trend among all soils where the simpler three ringed compound was degraded before the more complex five ringed PAH. Kennes et al (1994) found a similar trend where a two ringed PAH was degraded prior to a three ringed PAH. All seven soils experienced a lag period before B(a)P degradation occurred. Previous studies also have noted lag times of 28 days (Grosser *et al.*, 1991) and 52 days (Kanaly *et al.*, 1997) for B(a)P degradation to begin. This would suggest that either the same pathway or coordinated pathways were used to degrade PAHs and the simpler compounds were utilized by the microbial community prior to the more complex PAHs.

The K_d values in Table 4.7 indicated that these chemicals have the tendency to sorb to soil. Limited bioavailability of anthracene could be the reason for degradation activity leveling off at 40 to 50 % of the added radioactivity. Sorption of B(a)P could have been the reason for the low levels of degradation seen in four of the seven soils.

Anthracene and B(a)P are both known to have low vapour pressure (1.96×10^{-4} and 5.0×10^{-7} torr at 20°C) and high log K_{ow} values (4.45 and 6.04), therefore these compounds are not expected to volatilize (Sims and Overcash, 1983). The methanol extraction of the polyurethane foam plugs found that volatilization from the soils during the degradation study was not a major pathway of loss for the ^{14}C-labeled PAHs.

4.6.2 Biological Indicators of PAH Degradation

Respiration followed a similar trend to PAH degradation. Although there was variability in the data, the three contaminated soils that had the greatest ability to degrade the PAH compounds also had the highest rate of microbial activity. These were the two contaminated agriculture soils and the top layer of the industrial highly contaminated soil.

At the agriculture site the biomass carbon levels decreased as contamination increased. This suggests that the size of the microbial community had been impacted by the presence of the contamination yet the overall activity in these sites increased. The industrial site did not follow the same trend. Of the four soils at this site the top layer of the highly contaminated soil had the largest microbial biomass, the highest activity, and the greatest ability to degrade PAHs. This site had been subjected to

creosote contamination for a number of years and perhaps this had allowed the soil microbial communities to adapted and to reestablish a diverse microbial community. The contamination at the agriculture site occurred more recently than at the industrial site; therefore, the soil microbial communities may still have been recovering from the initial contamination. The more recent contamination may also have had a larger fraction of the hydrocarbon compounds available to the microbial communities as an additional carbon source.

Immediately after collecting our soil samples the metabolic diversity of the soil microbial biomass was assessed using the Biolog system. There were no significant differences in the metabolic diversity of the soils from the agriculture site. These results suggested that the impact of the crude oil contamination on these soils has not affected the ability of the microbial community to function. All three soils could utilize approximately the same percentage of the 95 different carbon substrates that were tested. Only the upper layer of the highly contaminated soil at the industrial site had a significantly higher metabolic diversity than the other three soils from this site. These results suggest that the highly organic top layer supports a microbial community with an increased ability to utilize the wide range of substrates found in these plates.

The metabolic diversity of a soil microbial community was not an indicator of the ability of a soil to degrade PAH compounds. There was no consistent trend between metabolic diversity and the ability of these soils to degrade PAHs. These results indicate that there were virtually no differences between the metabolic diversity

at each of these two sites though differences in their ability to degrade PAHs were seen.

Metabolic diversity of the microbial population was also determined on soils at the end of the degradation experiment. In both the anthracene and the benzo(a)pyrene set of soils the agriculture control soil and agriculture low level of contaminated soil had a much reduced metabolic diversity than was found prior to the degradation study. An increase in the variability between replications also was seen. This suggested that by the end of the 350 day degradation study the supply of carbon in these soils was limiting growth and maintenance of the microbial community. The metabolic diversity was lower for the agriculture highly contaminated soil than was seen prior to the degradation experiment, but this was not as large of a difference as was seen for the other two soils at this site. The high level of contamination in this soil may have resulted in a larger carbon supply to the microbial community than in the other two soils from this site.

The metabolic diversity of the industrial soils decreased by the end of the degradation study. In both sets of the ^{14}C-PAH contaminated soil the moderately contaminated soil had the lowest metabolic diversity of all the industrial sites. The top layer of the highly contaminated industrial soil had the highest metabolic diversity, which had slightly decreased from the initial assessment of metabolic diversity. Again, it was probably the carbon supply that was limiting the microbial communities and therefore resulted in lower metabolic diversity values by the end of the incubation period.

Assessment of the metabolic diversity of the uncontaminated soils that had been incubated for the same time period as the test soils found similar results as those found for the anthracene and B(a)P contaminated soils at the end of the degradation study. If the decrease in metabolic diversity displayed in the soils after the degradation study was due to the toxicity of the added diesel stock solutions then this would not have been seen in this additional set of microcosms. Since the same effect was seen in this set as was seen in the anthracene and benzo(a)pyrene set of microcosms, this suggests that during the incubation period carbon became limiting to the soil microbial community. The soils that had the highest respiration activity during the experiment maintained the highest metabolic diversity.

Complex PAH compounds are believed to be degraded by cometabolism where one substrate is used for energy and growth of the microorganisms while the pollutant is concurrently transformed without providing benefits to the microbial community (Bumpus, 1989; Glaser and Lamar, 1995; Kastner *et al.*, 1994; Pothuluri and Cerniglia, 1994; Wilson and Jones, 1993). The metabolic diversity data indicates that that available carbon supply became limiting to the soil microorganisms during the microcosm incubation. With limited substrate available to maintain the microbial community the cometablic pathways may not have been able to function therefore degradation of the complex PAH compounds may no longer have been possible by these pathways.

Degradation of B(a)P occurred after approximately a 140 day lag period. At this time the soil microbial community may have been experiencing stress due to the limited carbon supply of this closed system apparatus. Secondary metabolism occurs when primary growth is stopped due to a nutrient limitation (Kirk, 1985). Perhaps a nutrient

limitation in the soil induced a shift from primary to secondary metabolism that was responsible for the degradation of B(a)P.

The microcosm apparatus that contained the contaminated soils differs from the conditions experienced in a hydrocarbon spill site situation. The environment created by the microcosms is a closed system that would be more representative of a bioreactor where inputs and soil conditions can be strictly regulated. This experiment suggests that perhaps limiting the carbon supply in a bioreactor would induce degradation of resistant aromatic compounds.

4.6.3 Extractions and Wet Oxidation

For both the anthracene and B(a)P sets of soil only a small fraction of the radioactivity was incorporated into the microbial biomass. The complexity of these ringed compounds is thought to make them unsuitable for anabolic utilization. In order to obtain energy for growth and maintenance microorganisms may require the use of cometabolic degradation pathways for the breakdown of complex PAH compounds (Bumpus, 1989; Kastner *et al.*, 1994; Pothuluri and Cerniglia, 1994; Wilson and Jones, 1993).

Birkholz (1992) used a 100 % methanol extraction to remove compounds from hydrocarbon contaminated soils. The main classes of chemicals removed from the soil via this method were polycyclic aromatic sulfur heterocycles, polycyclic aromatic hydrocarbons, and saturated hydrocarbons. Methanol extraction detected approximately 2 to 12 % of the added ^{14}C-anthracene and 5 to 15 % of the added ^{14}C-B(a)P. This fraction consists of compounds that are weakly associated with the soil and

are thought to have limited bioavailable. PAH degradation intermediates that have been absorbed by the soil may be extracted by methanol. If the initial oxidation of anthracene had occurred at the unlabeled outside ring then $^{14}CO_2$ would not have evolved though degradation had taken place. These degradation products may be more polar than their parent compounds, but sorption to the soil would have resulted in an underestimation of total transformation of the PAH compounds that had taken place.

Total digestion of soil organic carbon by wet oxidation released the ^{14}C associated with the soil. This fraction of radioactivity may have been humified and is considered to be unavailable to the microbial community. Due to the hydrophobic nature and the low volatility of the two PAH compounds it was expected that this fraction would have been the largest. As mentioned previously Richnow (1995) performed a degradation study and found that 50 % of ^{14}C-labeled anthracene had become bound to the soil. It was thought that the labeled compound had become associated with the humic substances. A relatively small fraction of the added ^{14}C-labeled anthracene was detected by the wet oxidation method (< 16 %) while the amount of ^{14}C-labeled B(a)P detected in this fraction ranged from 10.6 % to 56.8 %. At the agriculture site the amount of soil associated radioactivity from B(a)P decreased as the level of contamination increased from the control to the highly contaminated soil. The moderately contaminated industrial soil had a significantly higher level of ^{14}C in this fraction than was found in the other three soils from this site. The amount of radioactivity released from these soils by the wet oxidation procedure is thought to have been underestimated. This is further discussed in section 6.8.

4.6.4 Total Recovery of ^{14}C

A large percentage of the added radioactivity was not accounted for by the series of extractions and the wet oxidation procedure. Total recovered ^{14}C-labeled anthracene ranged from 47.9 ± 4.0 % to 56.6 ± 1.5 % and benzo(a)pyrene ranged from 43.7 ± 14.1 % to 70.0 ± 6.7 %. The possible reasons for this are discussed further in chapter 6.

The major fate of anthracene in all seven of the soils was microbial degradation. The evolution of $^{14}CO_2$ was monitored for the duration of the experiment. Detection of $^{14}CO_2$ is considered to be the best evidence of mineralization activity yet a large portion of the radioactive compound may have an alternative fate such as incorporation into the biomass (Shuttleworth and Cerniglia, 1995). Due to the structure of anthracene and the location of the ^{14}C-labels there was a 50 % chance that the initial oxidation of the ring structure would occur at the labeled benzene ring. If ring fission occurred at the labeled ring then $^{14}CO_2$ would be produced early in the degradation process. If the initial oxidation occurred at the unlabeled ring then $^{14}CO_2$ evolution would be delayed until the first ring was removed and the two ringed degradation intermediate entered the naphthalene degradation pathway. Often uniformly labeled compounds are not commercially available. The labels are placed where the compounds are most likely to be converted to CO_2 (Shuttleworth and Cerniglia, 1995).

The major fate of B(a)P in three of the seven soils was microbial degradation. The other four soils, which experienced low levels of B(a)P degradation, had the majority of the ^{14}C-label associated with the soil. The two ^{14}C-labeled carbons in B(a)P were located on an outside ring that would be the most energetically favorable location for the

initial oxidation to occur (Carmichael and Pfaender, 1997). The evolution of $^{14}CO_2$ would indicate that the fission of this ring had taken place and degradation of B(a)P had begun. A substantial portion of the compounds may remain intact even after the removal of the ^{14}C-labels.

4.7 Summary and Conclusions

Previous exposure to hydrocarbon contamination affects the kinetics of anthracene and B(a)P degradation in soil. The most highly contaminated soils had a higher rate of initial anthracene degradation and a shorter lag time before B(a)P was degraded. The four soils that experienced low levels of B(a)P degradation had a large portion of the added radioactivity associated with the soil. In all cases the simpler PAHs were degraded before the more complex.

Respiration activity from the soils was the most consistent indicator of which soils had the greatest PAH degrading potential. The soils with the highest microbial activity had the most PAH degradation, though the variability in this data must be noted.

The metabolic diversity of soil microbial communities was affected over the long incubation period of this experiment. This was most likely due to a decrease in the carbon supply available to the microorganisms.

Soils that have been previously contaminated with hydrocarbons appear to have an increased ability to degrade B(a)P. At this time it is not clear if this is due to an adaptation of the microbial community to the presence of the contamination or if it is

due to differential expression of the metabolic pathways responsible for PAH degradation.

CHAPTER 5

Degradation of PAH Compounds in a Forest Soil

5.1 Abstract

The object of this study was to determine if a soil in which there is likely to be enhanced ligninolytic activity, such as in a Luvisolic soil under forest vegetation, could also degrade PAH compounds, such as B(a)P. The LFH, Ahe, and Ae horizons of a Luvisolic soil under aspen forest, collected in the Riding Mountain area were used for a degradation study with three ^{14}C-labeled PAH compounds: naphthalene, anthracene, and benzo(a)pyrene. The PAH compounds were added to the soils in a diesel stock solution. Degradation, volatilization, and respiration activity were monitored for the duration of the experiment.

The extent of naphthalene (2.9 to 3.3 %), anthracene (2.7 to 3.1 %), and benzo(a)pyrene (0.4 to 0.5 %) degradation were extremely low. An inoculum was prepared from a soil that displayed PAH degrading ability in a previous study and was added to the Luvisolic soil samples. This did not stimulate PAH degradation. Methanol extraction and wet oxidation procedures were performed on the soil at the end of the experiment in order to locate the ^{14}C-labeled compounds.

5.2 Introduction

Lignin degradation requires nonspecific enzyme systems that have been found to degrade xenobiotics. The onset of ligninolytic activity and pollutant degradation in fungal cultures has been found to occur simultaneously and under similar circumstances. It is these similarities that have led researchers to believe that persistent environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs), can degrade as a beneficial side reaction of ligninolytic activity (Hammel, 1992).

Ligninolytic activity is not regulated through common reactions of central metabolic pathways (Fenn *et al.*, 1981) but is suggested to be a process of secondary metabolism regulated by nitrogen (Fenn *et al.*, 1981; Fenn and Kirk, 1981; Keyser *et al.*, 1978; Kirk, 1984). The onset of ligninolytic activity responds to nitrogen depletion and is independent of the presence of lignin itself (Fenn and Kirk, 1981). This indicates that lignin degradation appears to be associated with a shift from primary to secondary metabolism (Keyser *et al.*, 1978).

Lignin peroxidases (LiP), manganese peroxidases (MnP), and laccases are ligninolytic enzymes which have been linked to the degradation of environmental pollutants (Bogan and Lamar, 1996). Hammel et al (1986) established a clear correlation between ionization potential and the suitability of certain PAHs compounds to be utilized as LiP substrate. It was determined that lignin related compounds undergo oxidation catalyzed by LiP as long as the compound has an ionization potential of less than or equal to 7.55 eV.

The complexity of a PAH compound may influence the ability of the microbial community to degrade that compound. The number of rings in the structure of a particular PAH is directly related to the stability of that compound. The inverse relationship between the number of rings in the structural formation and the ionization potential of an individual PAH may affect the ability of the microbial community to degrade these compounds.

5.3 Objective of the Study

The objective of this study was to determine if a Luvisolic soil occurring under forest vegetation, where ligninolytic activity is anticipated to be high, would result in enhanced degradation of three PAHs: naphthalene, anthracene, and B(a)P. The use of three PAH compounds with differing number of rings in the structural formation allowed an assessment of the role of chemical complexity in influencing the ability of the microbial community to degrade that particular compound.

5.4 Materials and Methods

5.4.1 Soil Description

A Luvisolic soil from the Granville association was collected from just south of Riding Mountain National Park (NE 29,17,16). Soils from the Granville Association range from very fine sandy loams to clay loams. These soils developed on boulder till of mixed limestone, shale, and granitic rock origin and are imperfectly drained (Ehrlich *et*

al., 1958). The area was dominated by aspen with sparsely distributed spruce trees. The LFH (3-5 cm), Ahe (0-3 cm), Ae (3-13 cm), and Bt (13-30 cm) horizons were sampled, stored in plastic bags, and kept at 4°C until needed (Table 5.1). The LFH varied in depth from approximately 3 to 5 cm depending on the location at which it was sampled. There were numerous roots throughout the Ahe horizon and its dark colour indicated the presence of organic matter. The Ae horizon was structureless and was light in colour. The Bt horizon had a definite blocky structure due to the translocation of clay from the upper horizons during soil development. A detailed soil description can be found in appendix II f.

5.4.2 Soil Properties

Soil characterization included determination of field capacity, pH, electrical conductivity, organic carbon, total nitrogen, and particle size distribution (Table 5.1).

Table 5.1 Soil properties of the Luvisol soil horizons.

Soil	pH	EC	Soil Moisture (%)	Field Capacity (%)	Organic Carbon (%)	Total Nitrogen (%)	Sand (%)	Silt (%)	Clay (%)	Textural Class	Sand Fraction				
											VCS	CS	MS	FS	VFS
LFH	5.8		67.6		39.14	1.17									
Ahe	5.6	0.178	23.6	59.6	8.99	0.33	39.9	34.7	25.3	L	3.7	9.5	18.2	29.5	26.7
Ae	5.2	0.144	8.7	15.8	0.50	0.07	59.2	15.3	25.5	SCL	4.8	11.2	20.6	33.7	29.8
Bt1	5.2	0.142	24.1		0.69	0.06	39.2	14.9	45.9	C	11.0	8.3	19.5	19.2	18.3
Bt2	5.0	0.231	22.6		0.41	0.17	39.2	13.7	47.0	C	15.5	12.9	19.8	28.7	23.1
Ck	7.3	0.225	17.1		0.50	0.10	49.5	22.2	28.3	SCL	27.3	13.2	18.5	24.2	16.9

5.4.2.1 Field Capacity. Field capacity for the Ahe and Ae horizons was determined by measuring the surface soil water content of soil columns brought to field capacity. A 15 cm long piece of plastic tubing with a diameter of approximately 5 cm had one end covered with cloth which was held firmly in place with an elastic band. Air dried soil from the Ae horizon that had been passed through a 2 mm sieve was added to within 2 cm of the open end of the tube. Water was added slowly and evenly over the surface of the soil until the water had moved one third of the way through the soil. The top of the apparatus was covered with Parafilm and allowed to sit for 48 hour at room temperature.

Ahe horizon contained a significant amount of roots. The field capacity tubes were filled with air dried soil from the Ahe horizon that contained the roots. The cloth was held in place at the bottom of the tube with an elastic band. Removal of the roots by sieving the air dried soil would have altered the nature of the horizon considerably. Addition of water to the top of this soil would have resulted in uneven distribution of the water due to paths created by the roots themselves. The tube of soil was placed on a tray of water and allowed to soak up water for 5 hours.

At the end of the time allowed for the distribution of water the moist soil was removed from the tube and placed into a previously weighed beaker. The moist soil was weighed before being placed in an drying oven at 110°C for 48 hours. A desiccator was used to cool the oven dried soil before the weight was determined. Gravimetric moisture content of the soil was calculated and is referred to as the field capacity of the soil.

5.4.2.2 Soil pH. Soil pH was determined by the $CaCl_2$ method outlined by Hendershot et al. (1993). Samples of a 1:2 ratio of air dried soil to 0.01 M $CaCl_2$ were prepared in 50 mL beakers. The solutions were mixed periodically for 30 minutes and were allowed to stand for 1 hour. The pH of the supernatant was measured by use of an Accumet® model 15 pH meter and was recorded once the pH had become relatively constant. The pH for each soil was determined in triplicate and averaged. Due to the high organic content of the LFH layer a 1:10 ratio of air dried LFH material to $CaCl_2$ was used. This is the ratio of soil to solution that is suggested for use with soil that contains high levels of organic matter.

5.4.2.3 Electrical Conductivity. Electrical conductivity of the Ahe and Ae horizons was determined. 10 g of air dried and sieved (2 mm) soil was weighed into 50 mL centrifuge tubes. Twenty milliliters of distilled water was added to each tube so there was a 1:2 ratio of soil to water. All tubes were sealed well and placed on a lateral shaker for 30 minutes. Immediately after the samples were removed from the shaker, an Orion model 160 conductivity meter was used to determine the electrical conductivity of soil solutions (McKeague, 1978).

5.4.2.4 Organic Carbon and Total Nitrogen. Organic carbon and total nitrogen of the soils were determined by a LECO CHN-600 combustion method (LECO Corporation, St. Joseph, MI). Carbonates had to be removed from the soil samples prior to the procedure. One gram soil samples were weighed into 30 mL beakers and 3 mL of 6 M

HCl was added to each beaker. The beakers were placed on a hot plate and covered with a watch glass. The solution was allowed to boil for 5 minutes; then it was removed from the hot plate and allowed to cool. The chloride from the acid had to be removed from the soil samples to avoid interference with the analysis of the LECO CHN-600. The soil samples were transferred to 50 mL centrifuge tubes and 25 mL of water was added to each tube. They were shaken by hand for two minutes and were placed in a centrifuge at 1500 rpm for 20 minutes. The supernatant was poured into a beaker and a few drops of $AgNO_3$ were added to the solution. If a AgCl precipitate occurred then chloride remained in the soil and the washing step was repeated. The washing step was repeated until the precipitate did not form.

The soil was transferred to a 50 mL beaker and placed in an oven at 105°C for a minimum of 24 hours. The samples were cooled in a desicator and finely ground with a mortar and pestle. A four decimal place scale was used to weigh approximately 0.1200 g of sample into a small metal container. The exact weight of the soil was recorded. All samples were run through the LECO CHN-600. The samples were fully combusted and the total nitrogen and organic carbon values were recorded.

5.4.2.5 Particle Size Distribution. Particle size distribution of the soil horizons, with the exception of the LFH layer, was determined by the pipette method as outlined by Sheldrick et al. (1993). The procedure was run in duplicate for each of the soils. The amount of sand, silt, and clay in the two samples was averaged before the texture class was determined. If the sand fraction accounted for greater than 30 % of the soil, the sand fraction was put through a set of sieves and separated into 5 size fractions.

5.4.3 Soil Preparation for Degradation Study

The top three horizons of the Luvisolic soil were chosen for a degradation study. Three sets of soil samples with four replications were prepared for the study for a total of 36 soil samples. The Ahe and Ae horizons had 10 g of soils on an oven dry basis weighed into 50 mL beakers. Due to the light weight of the LFH layer only 2 g sample on an oven dry basis was used for this experiment. The Ahe and Ae soil samples were wet to field capacity while the LFH samples were wet to 150 % moisture holding capacity. All samples were incubated at 20°C for approximately two weeks prior to the beginning of the experiment and they were incubated at this temperature for the duration of the study.

5.4.4 Microcosm Apparatus

The microcosm apparatus used to contain the soil samples consisted of a 500 mL glass jar with a tight fitting lid. In addition to the beaker of soil, the microcosms contained 15 mL of NaOH in a 20 mL scintillation vial to trap the CO_2, 3 mL of acidified water (pH 3) in a carrier vial to help maintain humidity, and a polyurethane foam plug suspended on a glass capillary tube to trap volatile compounds. Initially a 0.2 M NaOH solution was used to trap the CO_2 that evolved but due to the high rate of respiration activity from the LFH samples the concentration of NaOH used in the traps was increased to 0.5 M.

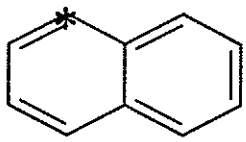
Approximately every two months the beakers of soil were removed from the microcosms, weighed, and rewet to field capacity if necessary. This was to keep the soils at a constant moisture content for the duration of the experiment.

5.4.5 ^{14}C-Labeled PAHs

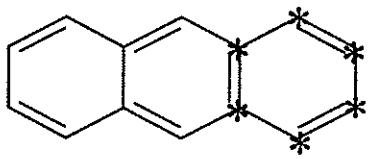
Three ^{14}C-labeled PAHs were purchased: ^{14}C-1-naphthalene (8.9 mCi/mmol at greater than 98% purity) and ^{14}C-1,2,3,4,4A,9A-anthracene (57 μCi/mmol at greater than 98% purity) from Sigma Chemical Company (St Louis, MO), and ^{14}C-7,10-B(a)P (12.2 μCi/mmol at greater than 98% purity) from Amersham International (Oakville, ON) (Figure 5.1). Stock solutions were prepared such that the addition of 1 mL to each beaker of soil would contain either 220 μg/g naphthalene, 100 μg/g anthracene, or 7.0 μg/g B(a)P, along with 5000 μg/g diesel fuel #2 and hexane. Hexane was a carrier solvent intended to aid in the distribution of the PAH compounds in the soil plug. ^{14}C-labeled and unlabeled PAHs were used to create the stock solution so that the level of activity added to each soil plug did not exceed 0.6 μCi. The level of naphthalene and B(a)P added to each soil plug was ten times the acceptable level for an industrial site according to the CCME Soil Quality Guidelines, March 1997. The level of diesel in the soil was such that Manitoba Environment would require remedial action to take place.

One milliliter of stock solution was added to each beaker of soil. The beakers remained in the fumehood for one hour after the addition of the PAH solution to allow the carrier solvent to volatilize. At the end of this period each beaker was weighed,

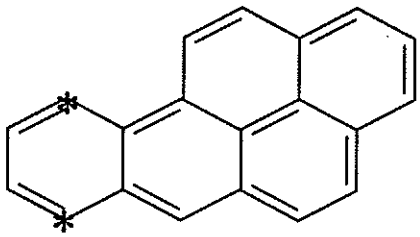
wet to field capacity, and placed into a microcosm apparatus. All microcosms had a scintillation vial containing 15 mL of 0.2 M NaOH added and were incubated at 20°C. There is an inverse relationship between the number of rings in a PAH and the loss from soil via volatilization. This removal mechanism becomes negligible for PAHs with greater than two rings (Davis *et al.*, 1993; Sims and Overcash, 1983; Wilson and Jones, 1993). It is not likely that the anthracene or B(a)P would have been lost from the soil samples during the time when the hexane was allowed to volatilize, though some of the naphthalene may have been lost at this time.



^{14}C-1-Naphthalene



^{14}C-1,2,3,4,4A,9A-Anthracene



^{14}C-7,10-Benzo(a)pyrene

Figure 5.1 Structural formulas of three PAH compounds: naphthalene, anthracene, and benzo(a)pyrene. The "*" represents the location of the ^{14}C-labels.

5.4.6 Inoculation

Six months after the experiment began, an inoculum was prepared from soils from a previous experiment (chapter 4: the industrial soil with a low level of contamination) that exhibited high levels of B(a)P degradation. The inoculum was added to the Luvisolic soils for the purpose of increasing PAH degradation. If the addition of the inoculum increased rates of degradation, then perhaps the indigenous microbial population of these soils did not possess the capability to perform the initial oxidation steps necessary for the breakdown of this recalcitrant compound.

A 10 g soil sample (on an oven dry basis) that had displayed B(a)P degradation was transfer to a dilution bottle containing 90 mL of saline-agar solution and glass beads. The bottle was placed on a lateral shaker for half an hour. Due to the thickness of this solution it was diluted with distilled water at a 1 to 1 ratio. A 1 mL aliquot of solution was used to inoculum the soil sample from each microcosm. The solution was distributed over the surface of each soil sample. Following the soil solution addition the beaker of soil was weighed, wet to field capacity, and returned to the microcosm. The evolution of $^{14}CO_2$ was monitored.

The soil sample used to create the inoculum contained ^{14}C-labeled B(a)P from a previous experiment. The level of radioactivity associated with a 1 mL sample of the soil solution was determined by use of a liquid scintillation counter and was taken into consideration during further calculations.

5.4.7 Monitoring Respiration and $^{14}CO_2$ Evolution

The NaOH traps in the microcosms were removed and replaced with new ones at 48 hours, 96 hours, and one week after the beginning of the incubation period. Weekly trap changes were done for the remainder of the experiment. After the first week of the experiment the concentration of the NaOH trap was increased from 0.2 M to 0.5 M due to the high respiration rates of the LFH layer.

A 1 mL sample was removed from each 15 mL NaOH trap and placed in a 7 mL scintillation vial for determination of $^{14}CO_2$ evolution. Each vial had 5 mL of EcoLume Liquid Scintillation Fluid (ICN Biochemicals Inc. Aurora, OH) added to it and was set aside for a minimum of 24 hours before the level of radioactivity was determined by a Beckman LS 7500 liquid scintillation counter. Calculations were done to determine the percentage of added radioactivity present in the entire 15 mL trap.

Total respiration was determined initially by performing a titration procedure on the remaining 14 mL of the NaOH traps. The vial containing each trap was emptied into a 100 mL titrator cup and rinsed well with distilled water. 5 mL of 0.5 M $BaCl_2$ was added to react with the carbon dioxide that was trapped by the NaOH resulting in the formation of $BaCO_3$ precipitate. 0.1 M HCl was used to titrate the unreacted NaOH from the trap. The volume of acid required for the titration was used to determine the amount of carbon dioxide that had evolved from the soil over the incubation period.

Two months into the experiment a modified version of the persulfate oxidation method for total organic carbon analysis (Technicon method # 455-76W/A) was used to

determine dissolved CO_2 and carbonates (Technicon, 1978). Dissolved carbon dioxide and carbonates were determined by acidifying the sample and passing carbon dioxide across a gas diffusion membrane. The carbon dioxide was then collected in a colour reagent containing 3.1×10^{-2} mmol of phenolphthalein per litre of solution and the change in colour was determined at 550 nm wavelength. The results were given in mg C/L of trap and then were converted into a mg C/trap basis.

5.4.8 Volatilization

At the end of the experiment the polyurethane foam plugs were removed from the microcosms and were placed in 100 mL French square bottles (EPA §796.3400). Twenty milliliters of methanol was added to each bottle. The bottles were sealed with rubber stoppers and were placed in a shaker on low for two minutes. A 60 mL syringe was used to extract all the methanol from the foam plugs. A 1 mL sample of the methanol extract was removed from each bottle, placed in a 7 mL scintillation vial with 5 mL of EcoLume scintillation cocktail, and was counted by a liquid scintillation counter. The amount of radioactivity trapped in the foam plugs was determined as a percentage of the radioactivity initially added.

5.4.9 Soil Microbial Biomass Carbon

The chloroform fumigation-extraction method was used to determine the soil microbial biomass carbon (Voroney *et al.*, 1993). Total organic carbon of the extract was determined by use of a persulfate oxidation method, Technicon method # 455-

76W/A (Technicon, 1978). Calculations were carried out as outlined by Voroney et al. (1993) using a K_c factor of 0.25.

5.4.10 Functional Diversity of the Microbial Community

Commercially available Biolog GN plates are composed of 96-wells that contain 95 different carbon substrates and a control well. These plates allow for a rapid method of characterizing and classifying microbial communities based on their ability to utilize sole carbon sources (Garland and Mills, 1991). Nutrients, salts, peptone, and redox dye tetrazolium violet are in all wells of the microplates. Tetrazolium dye is reduced in response to respiratory activity and results in the formation of formazan. The purple colour that results from this reaction is used as the response variable (Winding, 1994). It is thought that Biolog plates can be used to assess quickly and effectively the functional diversity of a microbial community (Zak *et al.*, 1994).

Biolog GN microplates were purchased through BIOLOG, Inc. (Hayward, CA). Two types of 100 mL dilution bottles were prepared: the first set contained 90 mL of water agar solution (0.2 %) which was prepared by dissolving 2 g of purified grade agar in 1 L of saline solution (0.85 % NaCl), and the second set contained 99 mL of physiological saline solution (0.85 %). Ten glass beads (5 mm in diameter) were added to each of the water agar bottles. All bottles were autoclaved prior to use, along with the pipette tips, and reagent reservoirs.

Ten grams of soil on an oven dry basis were weighed out in 4 replications for the Ahe, the Ae, and the Bt Luvisolic soil horizons. Due to low density of the LFH

horizon and the high number of microorganisms associated with this layer a 1 g sample of the LFH horizon was used for the Biolog plate assay. The samples were transferred by aseptic technique to the water agar dilution bottles, then the bottles were placed on a lateral shaker for a half hour. After shaking, 1 mL of the soil suspension was removed from each bottle and was added to a saline dilution bottle. The saline dilution bottles were shaken by hand. A multichannel pipette was used to place 100 µl aliquots of the final dilution of soil solution into each of the 96 wells in the Biolog plates. Biolog plates were incubated at 25°C and were removed for readings at 24, 48, 72, and 96 hours. At each reading the appearance of the reduced tetrazolium dye for each 24 hour period was noted. These readings were used to determine the metabolic diversity for each soil type. Metabolic diversity is defined as the percentage of substrates that were utilized by the microorganisms.

5.4.11 Soil Extraction and Wet Oxidation Procedure

At the end of the degradation study a 1 g sample of the LFH soils and a 5 g sample of the Ahe and the Ae soil (oven dry basis) were weighed into a Teflon centrifuge tube and were subjected to a methanol extraction procedure. The methanol extract would have contained ^{14}C-labeled PAHs and degradation intermediates that were weakly associated with the soil and are thought to have limited bioavailable. 20 mL of methanol was added to each centrifuge tube. All tubes were vortexed for 10 seconds and placed on a lateral shaker for 24 hours. The samples were then centrifuged at 1200 rpm for 20 minutes. A 1 mL sample of the methanol extract was removed for determination of the level of radioactivity associated with this fraction. Calculations were done to determine

the percentage of the added radioactivity that would be in the methanol extract if this procedure had been performed on the entire 10 g soil sample. The remainder of the methanol extract was poured out of the centrifuge tubes and the excess methanol was removed by drying the soil at 60°C for a minimum of 24 hours. The samples were removed from the oven and were finely ground with a mortar and pestle in preparation for the wet oxidation procedure.

A wet oxidation procedure was used to convert the total soil carbon to carbon dioxide (Voroney *et al.*, 1991). This allowed for any ^{14}C-labeled carbon that had become associated with the soil to be released and accounted for. A sample of the LFH layer (0.1 g) and the Ahe and Ae soils (0.4 g) were weighed into thick walled digestion tubes. A digestion mixture of CrO_3, concentrated H_3PO_4, and H_2SO_4 was prepared as outlined by Voroney et al. (1991). Six milliliters of the digestion solution was added to each tube. Immediately following this a glass rod was placed in each tube in order to support a 7 mL scintillation vial with 3 mL of 2 M NaOH for the LFH samples and 2 mL of 2 M NaOH for the Ahe and the Ae samples. The high level of carbon in the LFH layer requires that a smaller sample size and a larger volume of NaOH was used for the wet oxidation procedure to avoid having excess pressure in the digestion tube and saturating the CO_2 trap. A #49 Rubber Suba Seal was used to seal each tube tightly. In a fumehood the tubes were placed in the digestion block and were heated to 130°C for 1 hour. Once the digestion was complete, the apparatus was left to cool over night to allow for complete CO_2 adsorption. The CO_2 traps were removed from the digestion tubes and a 0.3 mL portion of the CO_2 trap was place in a 7 mL scintillation vial with 5 mL of Ecolume for determination of the radioactivity. The full volume of the base trap would be too

concentrated for the scintillation cocktail. Calculations were done to determine the percent of added radioactivity that was contained in the entire NaOH trap and the full amount of soil in the microcosm.

5.5 Results

5.5.1 Degradation Study

Degradation of the ^{14}C-labeled naphthalene initially was evident in all three soil horizons (Figure 5.2). Approximately two months into the experiment degradation leveled off at 2.9 to 3.3 % of the added radioactivity. Anthracene degradation experienced a short lag time before $^{14}CO_2$ production occurred from the soils (Figure 5.3). In all three soil horizons degradation was less than two percent until more than 100 days into the experiment and leveled off at between 2.7 and 3.1 %. By the end of the incubation period less than one percent of the ^{14}C-B(a)P had been recovered in the respiration traps. Due to the presence of impurities in the original ^{14}C-B(a)P stock solution, it is not certain that this small amount of ^{14}C-CO_2 was from B(a)P degradation (Figure 5.4).

Six months after the degradation experiment had begun an inoculum was added to the soils. Respiration activity and $^{14}CO_2$ production were monitored for two months following the inoculation. Microbial activity was present but the inoculation did not stimulate PAH degradation.

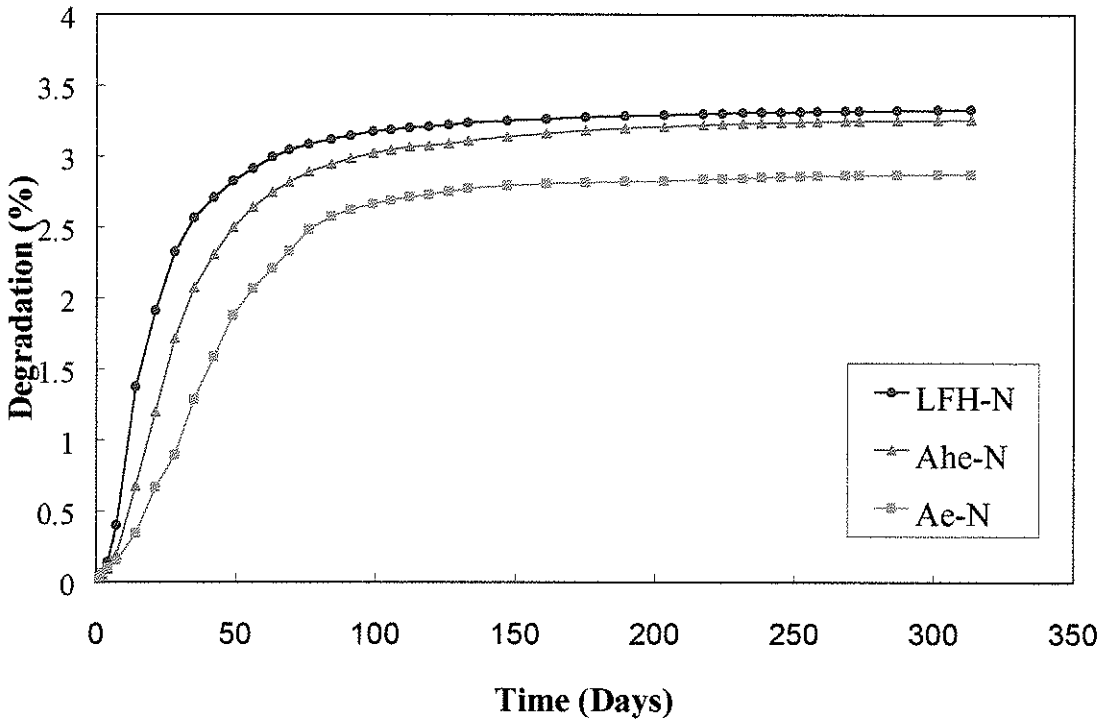


Figure 5.2 Cumulative degradation of naphthalene in the Luvisolic soil horizons. Points represent the mean of four replicates.

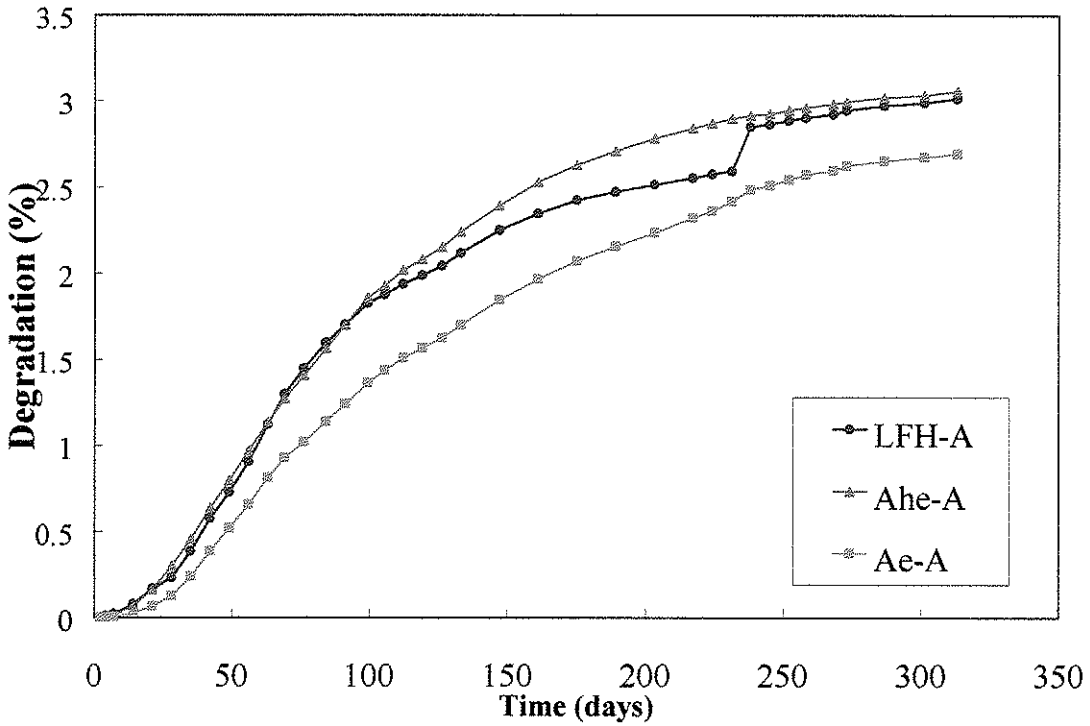


Figure 5.3 Cumulative degradation of anthracene in the Luvisolic soil horizons. Points represent the mean of four replicates.

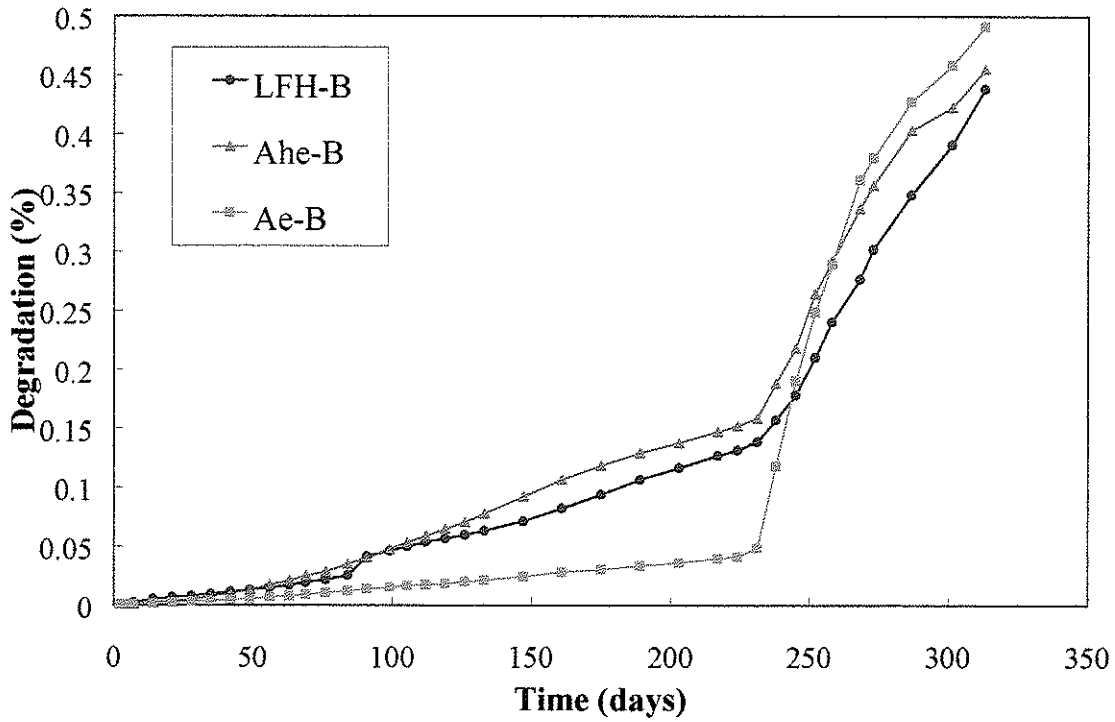


Figure 5.4 Cumulative degradation of benzo(a)pyrene in the Luvisolic soil horizons. Points represent the mean of four replicates.

Table 5.2 Recovery of the added ^{14}C-labeled PAHs. Values in the table are the mean of four replications ($\alpha = 0.05$).

Site	Degradation	Volatilization	Methanol Extract	Wet Oxidation	Total
Naphthalene					
LFH	3.3	0.01b	0.5	2.87b	6.7 ± 0.6
Ahe	3.3	0.01b	0.6	5.21a	9.1 ± 0.9
Ae	2.9	0.03a	0.7	2.34b	6.0 ± 0.3
	ns	*	ns	***	
Anthracene					
LFH	2.7	4.04b	2.6	25.14	34.5 ± 17.6
Ahe	3.1	3.36b	3.6	28.33	38.4 ± 2.0
Ae	2.7	6.08a	10.4	14.44	33.6 ± 12.6
	ns	*	ns	ns	
Benzo(a)pyrene					
LFH	0.4	0.00	41.2	37.46	79.1 ± 18.5
Ahe	0.5	0.00	8.2	22.58	31.3 ± 12.5
Ae	0.5	0.00	28.9	30.35	59.8 ± 17.9
	ns	ns	***	ns	

5.5.2 Respiration Activity

Respiration activity of the LFH layer was higher than from the Ahe and the Ae horizons in all three of the contaminated soils (Figure 5.5). The Ae horizon had the lowest amount of CO_2 evolution. The respiration data indicate that even if the added contamination had a toxic effect on some of the soil microorganisms, microbial activity was present during the incubation period even though PAH degradation was not evident.

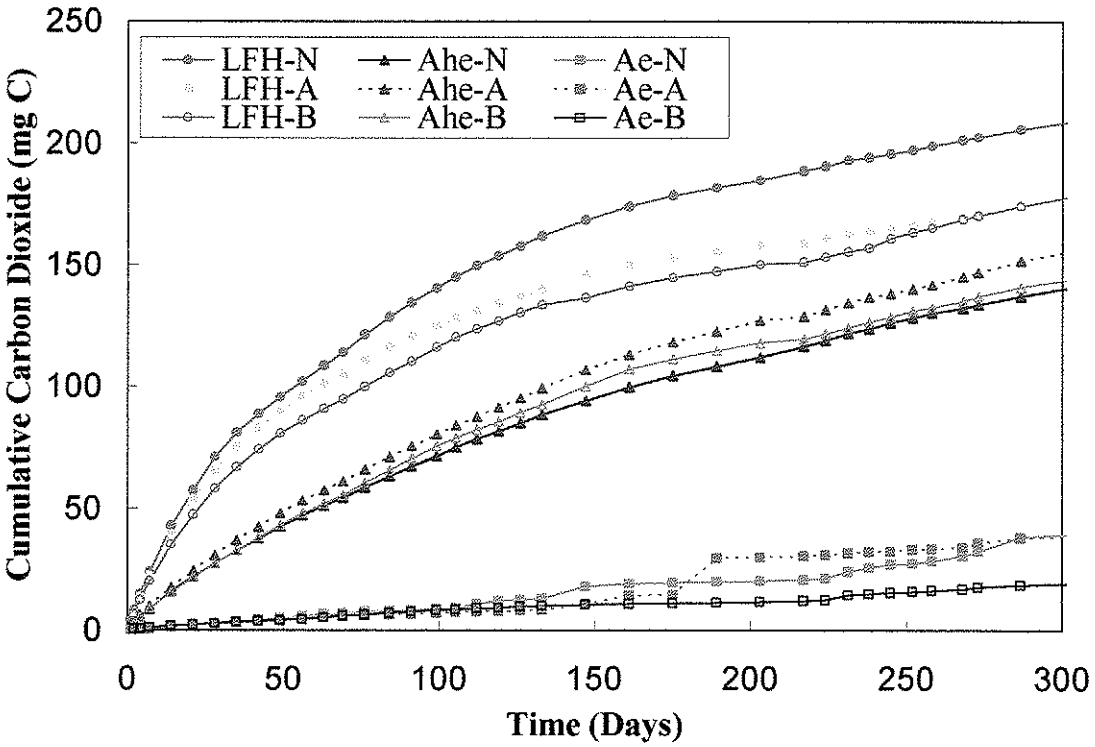


Figure 5.5 Cumulative respiration activity of a LFH, Ahe, and Ae horizon from a Luvisolic soil during the degradation study of naphthalene, anthracene, and benzo(a)pyrene. Points represent the mean of four replicates.

5.5.3 Volatilization

Methanol extraction of the polyurethane foam plugs indicated that volatilization of ^{14}C-anthracene or ^{14}C-anthracene derivatives had taken place from all three soil horizons (Table 5.2). The Ae horizon had a significantly higher level of radioactivity in the methanol extract than was found for the LFH layer and Ahe horizon. Less than one percent of the ^{14}C-labeled naphthalene that had been added to the soils was found associated with the methanol extract. Although the Ae horizon was found to have a significantly higher amount of volatilization, the overall level of radioactivity in all three horizons was low. The set of soils with the ^{14}C-B(a)P stock solution did not show any evidence of volatilization.

5.5.4 Microbial Biomass Carbon

The size of the microbial biomass decreased with depth in the soil profile (Table 5.3). The Ahe layer has a larger population size and a higher level of respiration activity than the Ae horizon. This is expected since the Ae horizon has less organic matter and a lower moisture holding capacity than the above Ahe horizon. The Ahe horizon would provide a better microbial environment than the Ae horizon. The microbial community of the LFH layer would be expected to be large due to the large pool of available carbon in this material (Swift *et al.*, 1979). Microbial biomass carbon was not calculated for the LFH layer since this layer contains a high amount of organic materials and would require a different methodology than that used for soil.

Table 5.3 Microbial biomass carbon from the Luvisolic soil horizons.

Soil Horizon	Microbial Biomass Carbon (μg/g)
LFH	nd
Ahe	1986.1 ± 297.6
Ae	435.9 ± 74.2
Bt_1	381.1 ± 218.5
Bt_2	70.2 ± 19.6
Ck	65.9 ± 1.7

Note: Microbial biomass carbon was not calculated for the LFH horizon. The "nd" indicates that there is no data present for this horizon.

5.5.5 Metabolic Diversity Study

A Biolog plate study of the Luvisolic soil indicated that there were significant differences in the metabolic diversity of the soil horizons (Table 5.4). The Ahe and the LFH were able to use the greatest percentage of the 95 substrates. The LFH and the Ae horizons were not found to be statistically different from each other while the Bt_1 was found to have the lowest metabolic diversity.

Table 5.4 Metabolic diversity of the soil microbial community found in the top four horizons from the Luvisolic site. Each value is a mean of four replicates ($\alpha = 0.05$).

Soil	Metabolic Diversity (%)
LFH	87.2% ab
Ahe	95.6% a
Ae	79.7% b
Bt_1	57.6% c
Significance	***

5.5.6 Extraction and Wet Oxidation Procedure

For the ^{14}C-labled naphthalene less than one percent of the added radioactivity was found in the methanol extract (Table 5.2). 2.6 % to 10.4 % of the radioactivity added as labeled anthracene was found in this fraction, but the amounts found in each horizon were not found to be significantly different from each other. The largest amount of ^{14}C associated with the methanol extracted fraction was from the ^{14}C-B(a)P set of soils. 41.2 % of the added radioactivity was found in the LFH layer of the Luvisolic soil. 8.2 % and 28.9 % was found with the Ahe and the Ae horizons respectively.

The wet oxidation procedure was intended to release any of the ^{14}C that was not removed from the soil samples by the methanol extraction procedure. The radioactivity that was released from the soil by digestion was thought to be strongly absorbed or incorporated into the soil (Table 5.2). A small fraction of the radioactivity from the ^{14}C-labeled naphthalene was detected by wet oxidation (2.34 % to 5.21 %). The ^{14}C associated with the Ahe horizon was found to be significantly higher than in the other two horizons. The high molecular weight ^{14}C-PAH compounds, anthracene and B(a)P, had a greater portion of the radioactivity detected by the wet oxidation procedure. The amount of ^{14}C from the labeled anthracene found in the LFH layer ranged from 11.6 % to 45.7 %. All three horizons from the ^{14}C-labeled B(a)P had a large range of variability in the level of added radioactivity detected in this fraction: LFH (20.5 % to 50.6 %), the Ahe (12.9 % to 41.0 %), and Ae (18.1 % to 58.1 %). There were no significant differences found between the ^{14}C-labled anthracene or B(a)P associated with each horizon.

5.6 Discussion

5.6.1 Degradation of PAHs

The nonspecific nature of ligninolytic enzymes has led researchers to believe that persistent environmental pollutants, such as PAHs, can degrade as a beneficial side reaction of ligninolytic activity (Hammel, 1992; Lamar *et al.*, 1990; Bumpus *et al.*, 1985). The LFH, Ahe, and Ae horizons of a Luvisolic soil were chosen for this study to examine the potential for PAH degradation in a system where ligninolytic activity is likely to be stimulated. Low levels of PAH degradation occurred in the Luvisolic soils over the incubation period. Although the naphthalene and anthracene contaminated soils exhibited measurable PAH degrading ability, the evolution of $^{14}CO_2$ was less than 5 % in all cases. The ^{14}C-labeled B(a)P did not degrade significantly, total degradation was less than 1 % by the end of the experiment. Due to the impurities present in the initial stock solution the low level of $^{14}CO_2$ evolution cannot be conclusively linked to the degradation of B(a)P. The addition of an inoculum prepared from a soil that displayed B(a)P degrading ability was unable to stimulate PAH degrading ability in these soils. It can be concluded that either the PAH compounds were not bioavailable at this time or that environmental conditions did not favour the expression of the required degradation pathways. If the inoculation of these soils had stimulated degradation of the PAH compounds, this would have suggested that PAH degrading microorganisms were not present in the soil prior to the inoculation. The results from this study imply that naphthalene, anthracene, and B(a)P are not degraded by ligninolytic systems.

The Luvisolic soil had not been exposed to hydrocarbon contamination prior to the introduction of the diesel stock solution for the degradation study. Sims et al. (1983) state that degradation of B(a)P can occur under natural conditions though breakdown is a function of soil concentration of B(a)P and previous exposure to PAHs. Non-acclimated soils showed low rates of B(a)P degradation if it was demonstrated at all. The microbial community in the Luvisolic soil horizons may require a period of time to adjust to the added contamination or a minimum concentration of PAH contamination to induce the metabolic pathways required for PAH degradation. The time period required for such an adjustment is not known, but an adaptation was not observed during the time course of this study. The contaminated soils from the agriculture site were sampled three years after the crude oil spill had occurred (Chapter 4). Degradation of B(a)P in these contaminated soils indicated that the enzyme pathways required for PAH degradation were being expressed. Kastner et al. (1994) suggested that PAH degrading bacteria may be present in a soil but a minimum concentration of PAHs may be required to stimulate growth of the PAH degrading microorganisms. The rate of addition in this study was such that each beaker of soil contained either 220 μg/g naphthalene, 100 μg/g anthracene, or 7.0 μg/g B(a)P. The PAH compounds were added in a diesel stock solution so the level of diesel contamination was 5000 μg/g of soil.

Carmichael et al. (1997) claimed that the initial oxidation of PAHs might be the rate limiting step of PAH degradation. They believed that as this initial process proceeds, then the PAH degradation intermediates become more readily available to a larger portion of the microbial community. Since PAH degradation was measured by monitoring the

evolution of $^{14}CO_2$, alteration of the parent compound that resulted in the production of early intermediate compounds other than carbon dioxide would not be detected.

Ligninolytic systems contain the laccase enzyme (Youn *et al.*, 1995). Laccase is thought to decrease the toxicity of pollutants by inducing polymerization reactions, which result in a decreased availability of these compounds. Its nonspecific nature results in its ability to couple pollutants with natural occurring compounds (Bollag *et al.*, 1988). Perhaps the PAHs in these soils have been oxidized initially but were incorporated into the soil. Field et al. (1992) referred to results reported by van der Trench and Sandermann in 1981, where the quinone form of B(a)P was found to be incorporated into lignin by horseradish peroxidase. Upon incorporation into the soil the compound may be less available to interact with the biota. This could result in a decreased toxicity of the parent compound and a reduced possibility of movement of the compound via leaching (Bollag, 1992; Bollag *et al.*, 1988; Richnow *et al.*, 1995; Shannon and Bartha, 1988). The wet oxidation results found that up to 45.7 % of the anthracene and 58.1 % of the B(a)P had become associated with the soil. This fraction of the ^{14}C-labeled PAHs might not have been bioavailable.

5.6.2 Respiration

Respiration data indicates that microbial activity was present during the incubation period even though there was limited degradation of the PAH compounds. The LFH layer had the highest level of activity but this did not result in an increased ability to degrade PAHs. Even after the addition of the inoculum respiration activity remained constant.

5.6.3 Volatilization

Significant loss of ^{14}C-labeled naphthalene and B(a)P was not detected by the methanol extraction of the foam plugs. Volatilization of B(a)P would not be expected due to its extremely low vapour pressure of 3.7×10^{-10} Pa at 25°C (Government of Canada, 1994). All three soil horizons had anthracene volatilize over the incubation period (3.4 to 6.1 %). The Ae horizon had significantly higher volatilization of anthracene occur than in the LFH layer and the Ahe horizon. The Ae horizon had a much lower organic carbon level than the other two soil horizons used in this experiment. Perhaps the lower level of organic matter resulted in less sorption of the PAH compound to the soil, allowing the anthracene or degradation intermediates to volatilize. Since anthracene and naphthalene have vapour pressures of 2.5×10^{-2} Pa and 11.960 Pa at 25°C respectively, naphthalene should have a greater tendency to volatilize. This suggests that perhaps the passive trap is underestimating the amount of naphthalene that volatilized during the experiment.

5.6.4 Metabolic Diversity

All three of the Luvisolic soil horizons used in this experiment had a high metabolic diversity, but none of these soils had the ability to degrade the PAH compounds. Metabolic diversity may be a good indicator of the ability of a soil microbial community to utilize a large range of substrates but it was not a good indicator of the ability of the soils to degrade PAH compounds.

In one of the four LFH Biolog plates there was a small portion of the control well that had colour development occur during the 72 to 96 hour incubation period. The

control well in each of the Biolog plates does not contain a carbon substrate. The formation of the purple precipitate would imply that the carbon substrate in the control well that resulted in respiration activity must have been in the LFH extract from the dilution procedure. At the time when the Biolog plate was inoculated with the LFH extract, the carbon must have been added.

5.6.5 Methanol Extraction and Wet Oxidation Procedure

Only a small portion of the ^{14}C-labeled naphthalene was recovered by the methanol and wet oxidation procedures (Table 5.2). The wet oxidation found a significantly higher amount of ^{14}C associated with the Ahe horizon than was found with the other two horizons, though the percentage of radioactivity recovered by this procedure is relatively small.

Birkholz (1992) used a 100 % methanol extraction to remove compounds from hydrocarbon contaminated soils. The main classes of chemicals removed from the soil via this method were polycyclic aromatic sulfur heterocycles, polycyclic aromatic hydrocarbons, and saturated hydrocarbons. A small portion of the radioactivity was recovered by methanol extraction of the anthracene contaminated set of soils. This fraction consists of compounds that are weakly associated with the soil and are thought to have limited bioavailablity. The ^{14}C-labeled PAH compound or its degradation intermediates may have been adsorbed by the soil and recovered by the methanol extraction. Although a larger portion of the radioactivity appears to be associated with the Ae horizon than the LFH layer and Ahe soil samples, values for these were not found to be statistically different.

The largest fraction of the added ^{14}C-anthracene was detected by the wet oxidation method. This fraction of radioactivity may have been humified and is considered to be unavailable to the microbial community. The level of radioactivity associated with the three horizons was not found to be significantly different.

A large portion of the ^{14}C-labeled B(a)P was detected in the methanol extract from the LFH layer (41.2 %). The Ae horizon also had a significant portion of the radioactivity associated with it (28.9 %) while the Ahe horizon had less than ten percent (8.2 %). Wet oxidation of the Luvisolic soil detected a large percentage of the ^{14}C-labeled B(a)P, although the amount of radioactivity associated with the three horizons was not found to be significantly different.

Due to the hydrophobic nature and the low volatility of anthracene and B(a)P it was expected that the wet oxidation fraction would have contained the largest amount of the ^{14}C-label. Soil sorption coefficients (K_d values) have been calculated for the three PAHs in the Luvisolic soil horizons (Appendix IV a) which indicate that anthracene and B(a)P would sorb strongly to the LFH layer and Ahe samples. K_d values are a function of the fraction of organic carbon; therefore, the highest tendency to sorb is to the LFH layer and then to the Ahe horizon. The Ae has a very low level of carbon, but the extreme hydrophobic nature of B(a)P leads to a high K_d value even for this horizon.

5.6.6 Total Recovery of ^{14}C

Total recovery of the added ^{14}C-labeled naphthalene was low (6.2 % to 10.2 %). Volatilization of the ^{14}C-naphthalene may have occurred following the addition of the diesel stock solution. The soils remained in the fumehood for one hour to allow the carrier solvent to evaporate. Some of the naphthalene may have volatilized along with the hexane. Volatilization of anthracene and B(a)P during the addition of the diesel stock solution does not seem likely due to their low vapour pressure at room temperature (1.96×10^{-4} and 5.0×10^{-7} torr at 20°C) and high $\log K_{ow}$ values (4.45 and 6.04) (Sims and Overcash, 1983). Further volatilization of naphthalene may have occurred during the experiment. The passive nature of the polyurethane foam plugs may not have been a suitable trapping device for this highly volatile compound. ^{14}C-labeled naphthalene (Figure 5.1) contains only one radioactive carbon in its structure. Complete ring fission must take place for ^{14}C-CO_2 to be produced.

Total recovery of the ^{14}C-labeled anthracene and B(a)P ranged from 19.7 % to 57.2 % and 20.0 % to 104.9 % respectively. The ^{14}C-labels in anthracene were contained in an outer ring (Figure 5.1). Due to the structure of anthracene and the location of the ^{14}C-labels there was a 50 % chance that the initial oxidation of the ring structure would occur at the labeled benzene ring. If ring fission occurred at the labeled ring, then $^{14}CO_2$ would be produced early in the degradation process. If the initial oxidation occurred at the unlabeled ring, then $^{14}CO_2$ evolution would be delayed until the first ring was removed and the two ringed degradation intermediate entered the naphthalene degradation pathway. When anthracene is degraded to form a two ringed compound,

resembling naphthalene, the volatility of the degradation product will be greater than anthracene. Losses may have occurred due to this increased volatility.

B(a)P is not a volatile compound as indicated by its very low vapor pressure. After initial oxidation of this compound the resulting degradation intermediates would be large in size and would be expected to have a small tendency to volatilize. The ^{14}C-labels are located in the ring that would be the first site of oxidation activity. Fission of this ring would lead to the formation of $^{14}CO_2$, which would be contained in the respiration trap. The hydrophobic nature of B(a)P results in its tendency to sorb to soil. Perhaps the wet oxidation procedure underestimated the amount of radioactivity associated with some of the soil samples. This is further discussed in section 6.8.

5.7 Summary and Conclusions

The indigenous microbial communities of the Luvisolic soil horizons did not degrade the three PAH compounds used in this experiment. Addition of an inoculum did not initiate PAH degradation. The results from this study imply that Luvisolic soils, which are expected to have an active ligninolytic systems, do not result in enhanced degradation of PAHs. The major fate of the ^{14}C-labeled anthracene and B(a)P in the Luvisolic soil horizons was to become associated with the soil. Since the level of PAH degradation was low in all three soil horizons, it was not possible to determine if the structural formation of an individual PAH compound influences the ability of the microbial community to degrade a particular PAH compound.

Carbon dioxide evolution indicated that microbial activity was present in the soils even though degradation of the PAH compounds was limited. The Luvisolic soil horizons had a high metabolic diversity. Although the soil horizons were able to utilize a high percentage of the substrates in the Biolog plates, this was not a good biological indicator of the ability of the soil to degrade PAHs.

Ligninolytic activity is expected to be high in a Luvisolic soil, but degradation of the PAH compounds did not occur during the incubation period. The Luvisolic soil had not been subjected to previous hydrocarbon contamination. In Chapter 4 the agriculture and the industrial site had been exposed to crude oil and creosote contamination, respectively. The highly contaminated soils from both of these sites could degrade B(a)P. There is an apparent need for prior exposure to hydrocarbon contamination for PAHs to degrade in a soil environment.

CHAPTER 6

General Discussion

The purpose of this study was to determine if soil microbial communities have the metabolic potential to degrade PAH contaminants and occur in environments suitable for the expression of these pathways. Naphthalene, anthracene, and B(a)P were three PAH compounds chosen to represent a range of PAH complexity in a study examining how slope position and soil type, previous exposure to hydrocarbon contamination, and environments where ligninolytic activity would be selected for, influence the degradation of PAHs in a soil environment.

6.1 Landscape Position and Soil Type

Soil samples taken along a catena provided a range of microenvironments that were influenced by the local hydrology and soil properties of each individual site. Landscape influences the moisture regime of soils which has a direct effect on organic matter content and aeration status of soil. Since the known degradation pathways of PAHs are dependent on the presence of oxygen, it was hypothesized that this would influence the processes that are able to occur throughout the landscape and that determine the persistence of these contaminants within the environment (Manilal and Alexander, 1991; Pothuluri and Cerniglia, 1994). Soils

with a high aeration status would be expected to display an increased ability to degrade PAHs. Degradation activity was present in two of the catena sites in the soils taken from the crest and the upper slope positions. The overall percent of $^{14}CO_2$ evolution was extremely low. B(a)P did not degrade in the wide range of uncontaminated Manitoba soils collected from the catena sites.

6.2 Ligninolytic Degradation of PAH Compounds

Ligninolytic systems are believed to degrade PAH compounds. Luvisolic soils have developed under forest vegetation. The deposition of large amounts of lignin-rich material would be expected to stimulate ligninolytic activity. A Luvisolic soil and three PAHs were chosen for this experiment so that the potential for PAH degradation in a system where ligninolytic activity was induced could be examined. Initially the Luvisolic soil horizons appeared to have a greater ability to degrade naphthalene than anthracene and B(a)P. Napthalene is the simplest of the three PAHs chosen for this experiment and was expected to degrade at a faster rate than either anthracene or B(a)P. Naphthalene degradation leveled out at approximately three percent. Limited degradation of all three PAH compounds occurred in the Luvisolic soil horizons. The data from this experiment suggests that a lignin-rich environment does not necessarily result in the enhanced degradation of introduced PAHs.

$^{14}CO_2$ evolution was used as an indicator of PAH breakdown. A large percentage of radioactivity from the ^{14}C-labeled B(a)P was located in the methanol extraction of the LFH layer and Ae horizon. This methanol extractable portion of

the radioactivity may have had limited bioavailability. The wet oxidation procedure detected a large fraction of the ^{14}C added as anthracene and B(a)P. This portion of the PAHs or the PAH degradation intermediates that have become associated with the soil are believed to be unavailable to the microbial community to degrade.

It has been hypothesized that the number of rings in the structural formation of an individual PAH may affect the ability of the microbial community to degrade that particular compound (Grosser *et al.*, 1991; Shuttleworth and Cerniglia, 1995; Sims and Overcash, 1983; Wilson and Jones, 1993). The limited degradation for all compounds studied prevented an evaluation of this hypothesis.

6.3 Previously Contaminated and Uncontaminated Soils

The catena soils and Luvisolic site had not been exposed to previous hydrocarbon contamination. Either the indigenous microbial communities of these soils did not have the ability to degrade a significant amount of the B(a)P or environmental conditions inhibited or failed to induce the expression of these pathways. To stimulate microbial activity the catena soils were dried and wet to field capacity (Kieft *et al.*, 1987; Sparling *et al.*, 1985), and were amended with carbon (Morgan *et al.*, 1993). Both of these treatments were unsuccessful at inducing B(a)P degradation. Kastner et al. (1994) suggested that PAH degrading bacteria may be present in a soil but a minimum concentration of PAHs may be required to stimulate growth of the PAH degrading microorganisms (Kastner *et al.*, 1994). Perhaps the concentration of B(a)P added to the soil in the diesel stock solution was not adequate to induce PAH degradation pathways (7 μg/g of soil).

The previously contaminated agriculture and industrial soils did degrade PAHs. The results from this study suggest that the rate of anthracene and B(a)P degradation is related to the level of previous hydrocarbon contamination. The three soils with the highest level of contamination initially were able to degrade the anthracene and experienced a shorter lag time before degrading B(a)P.

Anthracene degradation leveled off around 40-50 % for all soils with the exception of the moderately contaminated industrial soil. Richnow et al. (1995) found that approximately 50 % of the ^{14}C-labeled anthracene had become bound to the soil during a degradation experiment (Richnow *et al.*, 1995). The bioavailability of anthracene was limited due to sorption and the remainder of the anthracene was not available for degradation.

The highly contaminated agriculture soil was able to extensively degrade B(a)P. Over 50 % of the added radioactivity was recovered as $^{14}CO_2$. Three of the seven soils had greater than 30 % degradation of B(a)P. Previous research has reported extremely low levels, if any, degradation of B(a)P (Barclay *et al.*, 1995; Grosser *et al.*, 1991; Morgan *et al.*, 1993). Grosser et al. (1991) believed that the reason for the low level of B(a)P degradation was due to partitioning of B(a)P into the sediment limiting bioavailability.

The agriculture control soil had not been exposed to previous hydrocarbon contamination. This soil had a lag time of approximately three weeks for significant degradation of anthracene to occur and had less than 2 % degradation of B(a)P by the end of the incubation period. The delay in evolution of $^{14}CO_2$ from the labeled

anthracene may have been due to the adaptation period required by the microbial community to adjust to the presence of the hydrocarbon contamination. At the end of the study significant degradation of B(a)P had not occurred in this soil. The only difference between the agriculture control soil and the other two agriculture sites was that the control soil had not been previous exposure to hydrocarbon contamination. This implies that the microbial potential for degradation of B(a)P was present in the agriculture soils but it was not expressed in the uncontaminated control soil.

Soils that have been previously contaminated with hydrocarbons appear to have an increased ability to degrade B(a)P. Reasons for this increased ability are not clear at this time. It may be due to an adaptation of the microbial community to the presence of the contamination or to differential expression of the metabolic pathways responsible for PAH degradation.

6.4 Inoculation

The indigenous microbial communities of the catena and the Luvisolic soils lacked the ability to express the required pathways for degradation of B(a)P. An inoculum was prepared from a soil sample taken from the contaminated soil study that had displayed B(a)P degrading ability. The catena and the Luvisolic soils were inoculated. Since this did not induce microbial degradation, it can be concluded that either the PAH compounds were not bioavailable at this time or that environmental conditions did not favour the expression of these pathways. If degradation of the PAH compounds had resulted after the inoculation, then this would have suggested that the required microorganism were not present in the soil prior to inoculation.

6.5 Competitive Metabolism

Resent research has found there to be a competitive interaction that influences the order to which PAH compounds will degrade (Kanaly *et al.*, 1997; Shuttleworth and Cerniglia, 1996; Stringfellow and Aitken, 1995). The data from the contaminated soils degradation study supports the theory that simpler PAHs are degraded before the more complex PAH compounds. All five soils that were able to breakdown both anthracene and B(a)P degraded the three ringed anthracene prior to producing significant levels of $^{14}CO_2$ from the five ringed B(a)P. It has been suggested that this provides evidence of a shared enzyme pathway that exists to degrade PAHs (Stringfellow and Aitken, 1995). The presence of naphthalene and methylnaphthalenes has been found to interfere with the degradation of phenanthrene (Stringfellow and Aitken, 1995) and other high molecular weight PAHs (Kanaly *et al.*, 1997; Shuttleworth and Cerniglia, 1996). This would suggest that either the same pathway or coordinated pathways were used to degrade PAHs and the simpler compounds were utilized by the microbial community prior to the more complex PAHs.

6.6 Biological Indicator of PAH Degrading Ability

For the contaminated soils study microbial respiration proved to be the best indicator of PAH degradation. The soils with the highest level of microbial activity had the greatest ability to degrade PAHs. The hydrocarbon contamination present at this site may have resulted in an additional carbon source for the soil microbial community to utilize. The soils with the highest level of contamination at the two sites had the highest

respiration rates and the greatest ability to degrade PAHs. Respiration alone however is not an indicator of the ability to degrade PAHs. The LFH layer of the Luvisolic soil had the highest level of microbial activity but the Luvisolic soil horizons did not have significant $^{14}CO_2$ evolution, indicative of PAH degradation. The concentration of previous hydrocarbon contamination may be a primary indicator of which soils will have the ability to degrade PAHs. Respiration may be used as a secondary indicator for determining which of these contaminated soils will have the greatest ability to degrade PAHs.

The size of the soil microbial biomass did not follow a consistent trend with the ability of a soil to degrade PAHs. At the agriculture site the size of the microbial biomass decreased with an increase in the level of contamination, but at the industrial site the largest microbial biomass was found in the most highly contaminated soil. At both sites the soils with the highest level of contamination had the greatest ability to degrade PAHs. The size of the microbial biomass is not a good biological indicator of the potential of a soil to degrade PAHs.

The assessment of metabolic diversity using the Biolog system indicated that the metabolic diversity of a soil microbial community was related to the level of available carbon in that soil. Measures of metabolic diversity were performed on the contaminated soils prior to the degradation experiment and then once again with the soils from the microcosm after the degradation experiment was terminated. In both the anthracene and the B(a)P set of soils the agriculture control soil and agriculture low level of contamination soil had a much reduced metabolic diversity after the degradation study. The highly contaminated soil had a slight decrease in metabolic diversity by the end of

the degradation experiment. The high level of hydrocarbon contamination in this soil may have provided the microbial community with a carbon source therefore maintaining the metabolic diversity of the soil for the duration of the incubation. This suggests that by the end of the degradation study the supply of carbon in many of these soils was limiting growth and maintenance of the microbial community. A similar trend was seen in the soils from the industrial site. All four soils had a decrease in metabolic diversity by the end of the degradation study. There was a greater decrease observed in the soil with the low level of contamination than in the highly contaminated top layer.

6.7 Fate of PAHs

Degradation was a major fate for the PAH compounds in the contaminated soils study. The catena and Luvisolic soils that had not been previously contaminated with PAH compounds did not degrade the PAH compounds. The data from all three studies indicates that volatilization was not a major fate for PAH compounds. Anthracene and B(a)P are both known to have low vapour pressure and high log K_{ow} values therefore these compounds are not expected to volatilize (Sims and Overcash, 1983). Significant losses of ^{14}C-labeled naphthalene and B(a)P were not detected by the methanol extraction of the foam plugs. Anthracene volatilization from the Luvisolic soil horizons ranged from three to six percent. Since anthracene and naphthalene have vapour pressures of 2.5 $\times 10^{-2}$ Pa and 11.96 Pa at 25°C respectively, naphthalene should have a greater tendency to volatilize. This suggests that perhaps the passive trap is underestimating the amount of naphthalene that volatilized during the experiment.

The hydrophobic nature of PAH compounds results in their tendency to adsorb to soil (Davis *et al.*, 1993; Field *et al.*, 1995; Wilson and Jones, 1993). Bioavailability of PAHs in soil may be related to the time period that the contaminates have been present and the tendency for these compounds to sorb to soil organic matter (Shuttleworth and Cerniglia, 1995). Since the incubation period of these three experiments ranged from approximately 300 to 700 days, a large portion of the radioactivity was expected to have become associated with the soil. There was a lot of variability in the amount of radioactivity detected by the wet oxidation procedure. Perhaps the amount of radioactivity associated with the soil was underestimated by the wet oxidation. Problems associated with using this procedure are discussed in the following section.

6.8 Possible Error in Total Recovery of Radioactivity

Total recoveries of the added radioactivity from the soil ranged from approximately 6 % to 70 %. The lowest level of recovery was from the ^{14}C-labeled naphthalene (5.9 % to 10.2 %) while the total ^{14}C recovered from anthracene (19.7 % to 57.7 %) and B(a)P (20.0 % to 104.9 %) were much higher. There are many reasons for the low level of ^{14}C recovery that occurred from some of the soil samples.

Following the addition of the diesel stock solution to the soil samples the samples were allowed to sit in the fumehood for an hour so volatilization of the carrier solvent could take place. It is possible that some of the PAH compounds may have volatilized with the hexane. Naphthalene has an extremely higher vapor pressure than anthracene and B(a)P (Table 2.3). This suggest that volatile losses of naphthalene may have

occurred with the evaporation of the hexane, but loss of anthracene and B(a)P should not have occurred at this point.

In the Luvisolic soil and the contaminated soil experiments 15 mL of NaOH (0.1 to 0.5 M) was used to trap the carbon dioxide that evolved from the soils. A commercially available scintillation cocktail was used to determine the level of radioactivity associated with each respiration trap. Voroney et al. (1991) claim that premixed scintillation cocktails have 85 % efficiency for counting $^{14}CO_2$ absorbed in NaOH. Error could have occurred due to this efficiency level.

The NaOH respiration traps may have absorbed water from the air inside the microcosms. A slight increase in the volume of NaOH would not have been accounted for in the calculations; therefore, the amount of radioactivity would have been underestimated. During the trap change process a small amount of $^{14}CO_2$ may have been lost when the microcosms were temporally opened. Although these losses associated with each trap change may have been small, numerous trap changes took place over the duration of the entire experiment.

In the preparation stage for the wet oxidation procedure the methanol had to be removed from the soils. The soil samples were placed overnight in an oven at 60°C. Some carbon compounds may have volatilized during this time.

The passive nature of the polyurethane plugs may not have been efficient in trapping the PAHs or the degradation products that volatilized from the soils. Due to the volatile nature of naphthalene and the naphthalene-like degradation products that should have resulted from anthracene breakdown a higher level of volatilization was expected from these two PAHs.

Many of the carbon dioxide traps from the wet oxidation procedure had a white precipitate and/or crystals in solution. If the precipitate and crystals contained carbon then the ^{14}C-label that was released from the soil was not fully accounted for. The wet oxidation procedure would have underestimated the amount of radioactivity that had become associated with the soil.

7. SUMMARY AND CONCLUSIONS

Soils that have been previously exposed to hydrocarbon contamination have the ability to degrade PAH compounds such as B(a)P. The most highly contaminated soils had a higher rate of initial anthracene degradation and a shorter lag time before B(a)P was degraded. Degradation of PAH compounds is related to the initial concentration of the hydrocarbon contamination in the soil. In all cases the simpler PAH was degraded before the more complex, suggesting that either the same pathway or coordinated pathways were used to degrade PAHs. As simpler PAH compounds are removed from a soil system and the more recalcitrant compounds remain, there will be a change in the biotoxicity of the contamination.

B(a)P was not degraded by the soils from the catena sites. The soils collected for this study are representative of a wide range of Manitoba soils. This implies that uncontaminated Manitoba soils do not express the pathways required for the degradation of B(a)P. The Luvisolic soil horizons were not able to degrade the three PAH compounds used in this experiment. This suggests that the ligninolytic activity characteristic of these forest systems does not result in an enhanced capacity to degrade PAHs. Alternative enzyme systems must be responsible for the breakdown of PAHs. The catena and Luvisolic soils had not been previously exposed to hydrocarbon contamination and did not have the ability to degrade B(a)P. There is an apparent need

for soils to have prior exposure to hydrocarbon contamination in order for B(a)P to be degraded. There is an adaptation of the microbial community to the presence of hydrocarbons such as creosote and crude oil, that enables degradation of B(a)P to occur in a soil environment.

For the previously contaminated soils, respiration activity from the soils was the most consistent indicator of which soils had the greatest PAH degrading potential. The soils with the highest microbial activity had the most PAH degradation, though the variability of this data must be noted. Metabolic diversity and size of the microbial biomass were not consistent indicators of the PAH degrading ability of a soil. The metabolic diversity of a soil appears to be regulated by carbon availability. As the level of available carbon decreases so does the metabolic diversity of the soil microbial community.

Further study is needed to determine the fate of PAHs and their degradation intermediates. Research has suggested that aromatic compounds such as PAHs can be incorporated into the humus structure along with humic acids. Upon humification the compound is thought to be less available to interact with the biota, therefore decreasing the toxicity of the parent compound, and reducing the possibility of movement via leaching (Bollag, 1992; Bollag *et al.*, 1988; Richnow *et al.*, 1995; Shannon and Bartha, 1988). If the fate of PAHs in a soil system involves humification, then the stability of these complexes and the possibility of future release of hazardous pollutants have to be determined.

The data from the contaminated soils study supports previous findings of competitive metabolism. PAHs are degraded in the order of complexity but further study

is needed to determine if it is the same degradation pathway or coordinated pathways that are responsible for PAH breakdown. If the pathways are known, then the conditions of the soil environment can be altered to activate PAH degradation.

PAHs have a strong tendency to sorb to soil. The availability of these compounds to the microbial community is not well understood. A better understanding of sorption, partitioning, and desportion of PAHs in a soil system needed so that the fate of PAH in the environment can be determined.

The results from these studies indicate that previous exposed to hydrocarbon contamination enables PAHs to degrade in a soil system. The time required by the soil microbial community to adapt to the contamination or the mechanisms involved that allow for the expression of the PAH degradation pathways in a contaminated environment need to be determined.

8. CONTRIBUTION TO KNOWLEDGE

This study found that soils with previous exposure to hydrocarbon contamination have a dramatically increased ability to degrade B(a)P. Three of the contaminated soils were able to degrade more than 30 % of the added ^{14}C-labeled B(a)P. This organic compound is considered to be a recalcitrant organic compound and a potential health hazard. The soils with the highest level of hydrocarbon contamination had the greatest ability to degrade PAHs and experienced a shorter lag time before degradation occurred. B(a)P degradation did not occur in the uncontaminated catena soils, the Luvisolic soils, or the agriculture control soil. These findings suggest a gradual adaptation of the native microflora to the added PAHs. The time requirement for this acclimation and the mechanisms involved are unknown at this time.

There is an order to the degradation of PAHs that is dependent on the complexity of the individual compound. In the contaminated soils that degraded anthracene and B(a)P the simpler three ringed compound was degraded prior to the more complex five ringed PAH. These results suggest that either the same pathway or coordinated pathways were used to degrade the PAHs. At a hydrocarbon contaminated site the simpler PAH compounds will be removed first and the more complex compounds will remain in the soil. As this process continues the spectrum of biotoxicity of the contaminated soil will change. Complex carcinogenic compounds such as B(a)P will

remain in the soil until the simpler compounds are degraded.

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APPENDICES

I. Terminology from the Canadian System of Soil Classification

Mineral Horizons and Layers

A - This is a mineral horizon formed at or near the surface in the zone of leaching or eluviation of materials in solution or suspension, or of maximum in situ accumulation of organic matter or both. The accumulation of organic matter is usually expressed morphologically by a darkening of the surface soil (Ah), and conversely the removal of organic matter is usually expressed by a lightening of the soil color usually in the upper part of the solum (Ae). The removal of clay from the upper part of the solum (Ae) is expressed by a coarser soil texture relative to the underlying subsoil layers. The removal of iron is indicated usually by a paler or less red soil color in the upper part of the solum (Ae) relative to the lower part of the subsoil.

B - This is a mineral horizon characterized by enrichment in organic matter, sesquioxides, or clay; or by the development of soil structure; or by a change of color denoting hydrolysis, reduction, or oxidation. The accumulation in B horizons of organic matter (Bh) is evidenced usually by dark colors relative to the C horizon. Clay accumulation is indicated by finer soil textures and by clay cutans coating peds and lining pores (Bt). Soil structure developed in B horizons includes prismatic or columnar units with coatings or stainings and significant amounts of exchangeable sodium (Bn) and other changes of structure (Bm) from that of the parent material. Color changes include relatively uniform browning due to oxidation of iron (Bm), and mottling and gleying of structurally altered material associated with periodic reduction (Bg).

C - This is a mineral horizon comparatively unaffected by the pedogenic processes operative in A and B, (C), except the process of gleying (Cg), and the accumulation of calcium and magnesium carbonates (Cca) and more soluble salts (Cs, Csa). Marl, diatomaceous earth, and rock no harder than 3 on Mohs'scale are considered to be C horizons.

Lowercase Suffixes

c - A cemented (irreversible) pedogenic horizon. Ortstein, placic, and duric horizons of Podzolic soils, and a layer cemented by $CaCO_3$ are examples.

ca - A horizon of secondary carbonate enrichment in which the concentration of lime exceeds that in the unenriched parent material. It is more than 10 cm thick, and its $CaCO_3$ equivalent exceeds that of the parent material by a least 5 % if the $CaCO_3$ equivalent is less than 15 % (13 % vs 8 %), or by at least 1/3 if the $CaCO_3$ equivalent of the horizon is 15 % or more (28 % vs 21 %). If no IC is present, this horizon is more than 10 cm thick and contains more than 5 % by volume of secondary carbonates in concretions or in soft, powdery forms.

e - A horizon characterized by the eluvation of clay, Fe, Al, or organic matter alone or in combination. When dry, it is usually higher in color value by one or more units than an underlying B horizon. It is used with A (Ae).

g - A horizon characterized by gray colors, or prominent mottling, or both, indicative of permanent or periodic intense reduction. Chromas of the matrix are generally 1 or less. It is used with A and e (Aeg); B alone (Bg); B and f (Bfg, Bgf); B, h, and f (Bhfg); B and t (Btg); C alone (Cg); C and k (Ckg); and several others. In some reddish parent materials matrix colors of reddish hues and high chromas may persist despite long periods of reduction. In these soils, horizons are designated as g if there is gray mottling or marked bleaching on ped faces or along cracks.

h - A horizon enriched with organic matter. It is used with A alone (Ah), or with A and e (Ahe), or with B alone (Bh), or with B and f (Bhf).

j - This is used as a modifier of suffixes e, f, g, n, and t to denote an expression of, but failure to meet, the specified limits of the suffix it modifies. It must be placed to the right and adjacent to the suffix it modifies. For example, Bfgj means a Bf horizon with a weak expression of gleying; Bfjgj means a B horizon with weak expression of both f and g features.

k - Denotes the presence of carbonate as indicated by visible effervescence when dilute HCl is added. It is used mostly with B and m (Bmk) or C (Ck) and occasionally with Ah or Ap (Ahk, Apk), or organic horizons (Ofk, Omk).

m - A horizon slightly altered by hydrolysis, oxidation, or solution, or all three to give a change in color or structure, or both. It has;

1. Evidence of alteration in one of the following forms;
    a. Higher chromas and redder hues than the underlying horizons.
    b. Removal of carbonates either partially (Bmk) or completely (Bm).
    c. A change in structure from that of the original material.
2. Illuviation, if evident, too slight to meet the requirements of a Bt or a podzolic B.

3. Some weatherable minerals.
4. No cementation or induration and lacks a brittle consistence when moist.

This suffix can be used as Bm, Bmgj, Bmk, and Bms.

n - A horizon in which the ratio of exchangeable Ca to exchangeable Na is 10 or less. It must also have the following distinctive morphological characteristics: prismatic or columnar structure, dark coatings on ped surfaces, and hard to very hard consistence when dry. It is used with B as Bn or Bnt.

p - A horizon disturbed by man's activities such as cultivation, logging, and habitation. It is used with A and O.

s - A horizon with salts, including gypsum, which may be detected as crystals or veins, as surface crusts of salt crystals, by depressed crop growth, or by the presence of salt-tolerant plants. It is commonly used with C and k (Csk), but can be used with any horizon or combination of horizon and lowercase suffix.

t - An illuvial horizon enriched with silicate clay. It is used with B alone (Bt), with B and g (Btg), with B and n (Bnt), etc.

L, F, and H - These are organic horizons that developed primarily from the accumulation of leaves, twigs, and woody materials with or without a minor component of mosses. Usually they are not saturated with water for prolonged periods.

(Agriculture Canada, 1987)

II a. Ryerson association soil description.

Horizon	Depth (cm)	Description
Crest Position		
Apk	0-10	Very dark grayish brown (10YR 3/1.5 m) loam, carbonates
Cca	10-18	Pale brown (10YR 6/3 m) clay loam, carbonates
Ck	18-40	Yellowish brown (10 YR 5/4 m) clay loam, carbonates
Upper Mid-Slope Position		
Ap	0-17	Black (10YR 2/1 m) loam
Bm1	17-25	Dark brown (10YR 3/3 m) loam
Bm2	25-38	Very dark grayish brown (10YR 3/2 m) clay loam
Cca	38-48	Very pale brown (10YR 7/3 m) silty clay loam, carbonates
Ck	48-65	Clay loam, carbonates
Lower Mid-Slope Position		
Ap	0-17	Black (10YR 2/1 m) clay loam
Ahe	17-21	Dark grayish brown (10YR 3.5/2 m) clay loam
Btgj	21-50	Very dark grayish brown (10YR 3/1.5 m) clay loam
Ccagi	50-75	Pale brown (10YR 6/3 m) silty clay loam, carbonates
Ckgs	75-90	Clay loam, carbonates
Toe Position		
Ap1	0-15	Grayish brown (10YR 4.5/1.5 m) clay loam
Ah(p)2	15-30	Black (10YR 2/1 m) clay loam
Aeg	30-40	Graysih brown (10YR 4.5/2 m) clay loam, mottles
Btg1	40-60	Clay, mottles
Btg2	60-80	Clay
BC	80-90	Clay

These are fine loamy soils that are moderately to very strongly calcareous. The parent material is till. The landscape is undulating and is presently used as cropland.

II b. Erickson association soil description.

Horizon	Depth (cm)	Description
Crest Position		
Ap	0-15	Dark grayish brown (10YR 4/1.5 m) clay loam
Btj	15-25	Dark brown(10YR 3/3 m) clay loam
BC	25-40	Brown (10YR 4.5/3 m) clay loam, carbonates
Cca	40-60	Very pale brown (10YR 7/3 m) clay loam, carbonates
Ck	60-80	Yellowish brown (10YR 5/4 m) clay loam, roots, carbonates
Upper Mid-Slope Position		
Ap	0-15	dark grayish brown (10YR 4/1.5 m) clay loam
Btj	15-38	Dark brown (10YR 3/3 m) clay loam
BC	38-45	Dark brown (10YR 3.5/2.5 m) clay loam, carbonates
Cca	45-70	Very pale brown (10YR 7/3 m) clay loam, carbonates
Ck	70-80	Yellowish brown (10YR 5/4 m) clay loam, carbonates
Lower Mid-Slope Position		
Apk	0-15	Black (10YR 2/1 m) clay loam
Ahk	15-40	Very dark grayish brown (10YR 2.5/1.5 m) clay loam, mottles, carbonates
Ccagj	40-60	Light brownish gray (10YR 6/2 m) clay loam, mottles, carbonates
Ckgj	60-75	Light yellowish brown (10YR 6/4 m) clay loam, mottles, carbonates
Toe Position		
Aph	0-15	Black (10YR 2/1 m) clay loam, mucky, carbonates
Ahk1	15-30	Black (10YR 2/1 m) clay loam, carbonates
Ahk2	30-50	Black (10YR 2/1 m) clay loam, carbonates

These are fine loamy soils that are moderately to very strongly calcareous. The parent material is till. The upper mid-slope, lower mid-slope, and toe position are slightly stoney (0.01-0.1). The landscape is undulating and is presently used as cropland. The crest and the upper mid-slope positions are well drained while the lower mid-slope and toe positions are imperfectly and poorly drained respectively.

II c. Waitville association soil description.

Horizon	Depth (cm)	Description
Crest Position		
Ap	0-15	Gray (10YR 5.5/1 m) clay loam
AB	15-18	Light gray (10YR 6/1 m) clay loam
Bt1	18-40	Very dark brown (10YR 2/2 m) clay, clay films
Bt2	40-65	Very dark grayish brown (10YR 3/2 m) clay, clay films
BC	65-75	Brown (10YR 4/2.5 m) clay loam, roots, carbonates
Upper Mid-Slope Position		
Ap	0-15	Grayish brown (10YR 5/1.5 m) clay loam
Bt	15-40	Dark brown (10YR 3/3 m) clay, clay films
Cca	40-55	Very pale brown (10YR 6.5/3 m) clay loam, mottles, carbonates
Ck	55-75	Grayish brown (10YR 5/2 m) clay loam, mottles, carbonates
Lower Mid-Slope Position		
Ap	0-15	Grayish brown (10YR 4.5/1.5 m) loam
Ahe	15-20	Very dark grayish brown (10YR 2.5/1.5 m) clay loam, mucky
Btgj	20-35	Very dark grayish brown (10YR 3/2 m) clay loam
BC	35-40	Dark yellowish brown (10YR 4/4 m) clay loam, mottles, carbonates
Ccagj	40-50	Dark grayish brown (10YR 4/2 m) clay loam, mottles, carbonates
Ckgj	50-70	Light brownish gray (10YR 5.5/2 m) clay loam, mottles, carbonates
Toe Position		
Apk	0-15	Very dark grayish brown (10YR 2.5/1.5 m) clay loam, mucky, carbonates
Ahk	15-35	Very dark grayish brown (10YR 3/1.5 m) clay loam, mottles, carbonates
Ccag	35-60	Very pale brown (10YR 7/3 m) silty clay loam, mottles, carbonates
Ckg	60+	Light brownish gray (10YR 6/2 m) clay loam, mottles, carbonates

These are fine loamy soils that are moderately to very strongly calcareous. The parent material is till. The landscape is hummocky and is presently used as cropland. The upper mid-slope position is moderately well drained, the lower mid-slope position is imperfectly drained, and the toe position is poorly drained.

II d. Newdale association soil description.

Horizon	Depth (cm)	Description
Crest Position		
Ahp	0-10	Very dark gray to very dark grayish brown
AC	10-23	
Ck	23+	
Upper Mid-Slope Position		
Ahp	0-15	Very dark gray to very dark grayish brown
AC	15-30	
Ck	30+	
Lower Mid-Slope Position		
Ah	0-30	Black to dark gray
Bm	30-45	Dark brown
Cca	45-55	
Ck	55+	
Toe Position		
Ah	0-45	
Bm	45-100	
BC	100-110	
Ckg	110+	

These are loamy soils that are moderately to strongly calcareous. The parent material is morainal till. The landscape is presently used as cropland. The crest position is moderately well to well drained, the upper mid-slope position is moderately well to well drained, and the lower mid-slope is well drained.

II e. Description of soils sampled at the agriculture site.

Horizon	Depth (cm)	Description
Control		
Ap	0-26	Lacustrine parent material
AC	26-40	Lacustrine parent material
Ckgj	40-95	Lacustrine parent material, gypsum at 40-50cm
CkgjII	90-120	Till parent material
Low Level of Contamination		
Apk	0-70	Weakly carbonated at surface
Ckgj	70-80	
Ckg	80-120	Weak evidence of gypsum, sand content increased at 100-110cm and return to moderately fine lacustrine materials at 110-120cm
High Level of Contamination		
Apk	0-18	Lacustrine parent material
AC	18-32	Lacustrine parent material
Ckgjs	32-90	Lacustrine parent material
Ckgs	90-120	Lacustrine parent material, gypsum at 70-90 cm

II f. Orthic Gray Luvisolic soil description.

Horizon	Depth (cm)	Description
LFH	5-0	Leaf litter layer, acidic
Ahe	0-3	Dark gray (10YR 4/1 m) lots of roots, acidic
Ae	3-13	Light brownish gray (10YR 6.5/2 m) signs of leaking
Bt1	13-30	Brown (10YR 4/3 m) acumulation of clay, angular blocky, acidic
Bt2	40-52	Brown (10YR 4/3 m) angular blocky, acidic
Ck	52+	Brown (10YR 5/3 m) carbonates

These soils develop on till parent material. The region is presently under forest vegetation that is dominated by aspen.

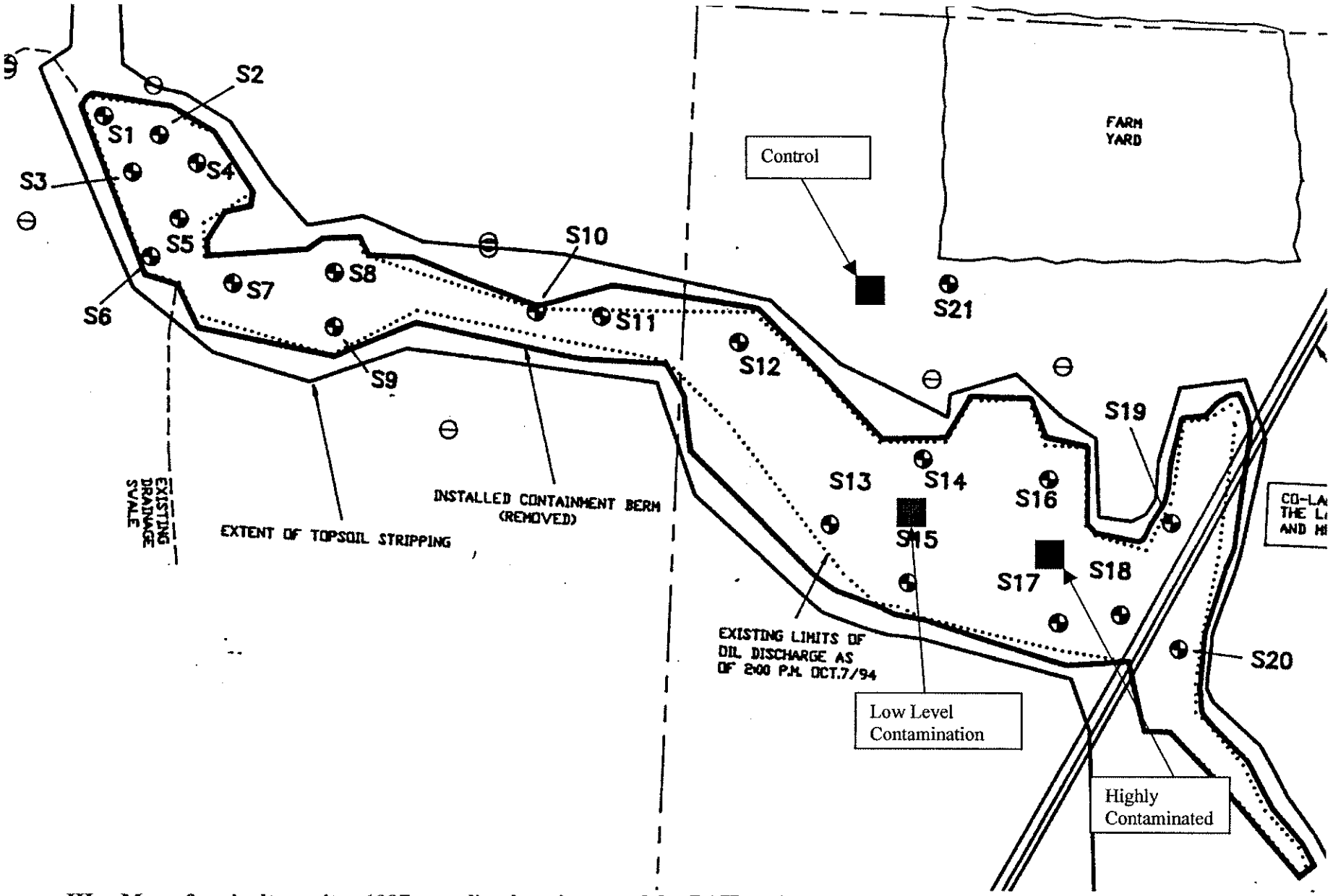
III a. PAH analysis of 1997 sampling of the agriculture and the industrial contaminated soils and diesel fuel used to create stock solutions: agriculture control (AC), agriculture low contamination (AL), agriculture high contamination (AH), industrial highly contaminated top layer (DHT), industrial highly contaminated bottom layer (DHB), industrial moderately contaminated (DM), and industrial low level of contamination (DL).

Sites	AC	AL	AH	DHT	DHB	DM	DL	Diesel
Naphthalene	<0.1	<0.1	<0.1	0.6	0.1	0.8	<0.1	246
Acenaphthylene	<0.1	<0.1	<0.1	2.7	0.3	1.4	0.1	8.4
Acenaphthene	<0.1	<0.1	<0.1	0.3	<0.1	0.1	<0.1	15.1
Fluorene	<0.1	<0.1	<0.1	0.3	<0.1	0.1	<0.1	48.2
Phenanthrene	<0.1	<0.1	<0.1	3.7	0.2	1.6	0.1	110
Anthracene	<0.1	<0.1	<0.1	4.1	0.6	1.6	0.2	9.6
Fluoranthene	<0.1	<0.1	<0.1	17.1	3.7	7.1	0.6	20.8
Pyrene	<0.1	<0.1	<0.1	17.3	4.1	7.4	0.6	94.2
Benzo(a)anthracene	<0.1	<0.1	<0.1	7.8	1.4	4.2	0.4	0.6
Chrysene	<0.1	<0.1	<0.1	12	2.1	6.3	0.6	2.4
Benzo-(b&k)-fluoranthenes	<0.1	<0.1	<0.1	19	2.7	10.3	1.1	0.7
Benzo(a)pyrene	<0.1	<0.1	<0.1	7.2	1	4.4	0.4	<0.5
Indeno(1,2,3-c,d)pyrene	<0.1	<0.1	<0.1	4.3	0.7	3	0.4	<0.5
Dibenzo(a,h)anthracene	<0.1	<0.1	<0.1	1.1	0.2	0.9	0.1	<0.5
Benzo(g,h,I)perylene	<0.1	<0.1	<0.1	3.8	0.5	2.6	0.3	11.7
Total PAH	ND	ND	ND	101.3	17.6	51.8	4.9	567.7

Units for PAH analysis in soil are mg/kg (ppm) and for PAH analysis in diesel is mg/L
ND - not within the detection limit

III b. PAH analysis from 1995 sampling of agriculture soils from an additional study. Map of sampling locations III c.

1995 Sampling	S13	S14	S15	S16	S17	S21
Benzo(a)anthracene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Benzo(a)pyrene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Benzo(b)fluoranthene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Benzo(k)fluoranthene	N.D.	N.D.	N.D.	N.D.	0.015	N.D.
Dibenz(a,h)anthracene	N.D.	N.D.	N.D.	0.014	0.02	N.D.
Indeno(1,2,3-c,d)pyrene	N.D.	N.D.	N.D.	0.011	0.016	N.D.
Naphthalene	0.064	N.D.	N.D.	0.18	0.43	N.D.
Phenanthrene	0.037	N.D.	N.D.	0.053	0.096	N.D.
Pyrene	0.16	0.011	N.D.	0.28	0.6	N.D.
Total PAHs	0.261	0.011	N.D.	0.538	1.177	N.D.
Total Purgeable Hydrocarbons (<C10)	240	ND	ND	170	200	ND
Total Extractable Hydrocarbons (C11-C30)	5200	120	ND	5200	4800	ND
Total Petroleum Hydrocarbons (<C30)	5440	120	ND	5370	5000	ND
Total Hydrocarbons (<C60)	5920	136	ND	5880	5450	ND



III c Map of agriculture site. 1997 sampling locations used for PAH analysis in relation to 1995 sampling locations.

IV a. Soil sorption partition coefficients (K_d) for the PAH compounds and soils used in the experiments. Kd values calculated based on equations by Karickhoff, 1981

Site	Naphthalene ($logK_d$)	Anthracene ($logK_d$)	Benzo(a)pyrene ($logK_d$)
Erickson Association Upper Mid-Slope			3.93
Waitville Association Upper Mid-Slope			3.57
Newdale Association Upper Mid-Slope			4.28
Newdale Association Depression			4.20
Ryerson Upper Mid-Slope			4.03
Agriculture Control		2.72	4.09
Agriculture Low Level Contamination		2.79	4.16
Agriculture High Level Contamination		2.82	4.19
Industrial Low Contamination		2.86	4.23
Industrial Moderate Contamination		3.47	4.84
Industrial High Top		3.41	4.78
Industrial High Bottom		2.86	4.23
LFH	2.57	3.75	5.12
Ahe	1.93	3.11	4.48
Ae	0.67	1.85	3.22